"The larger the island of knowledge, the longer the shoreline of wonder."

(*RW Sockman*)

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Antibiotic resistance transfer during
food production and preservation

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Titel van het doctoraat in het Nederlands: Overdracht van antibioticumresistentie tijdens voedselproductie en –bewaring.

Cover: Image of transconjugants by fluorescence microscopy

Notation index

AGP	Antibiotic Growth Promoter
AHL	Acyl Homoserine Lactones
AMCRA	Center of expertise on Antimicrobial Consumption and Resistance in Animals, Merelbeke, Belgium
BHI	Brain Heart Infusion
CFU	Colony Forming Unit
CI	Chromosomal Integron
CLSI	Clinical and Laboratory Standards Institute, Pennsylvania, USA
CRISPR/Cas	Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), and associated proteins (Cas)
CS	Conserved Segment
DAEC	Diffusely Adherent *E. coli*
DGGE	Denaturing Gradient Gel Electrophoresis
EAEC	Enteroaggregative *E. coli*
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control, Stockholm, Sweden
EEA	European Economic Area
EFSA	European Food Safety Authority, Parma, Italy
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMEA	European Medicines Agency, London, UK
EPEC	Enteropathogenic *E. coli*
EPS	Extracellular Polymeric Substances
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing, Växjö, Sweden
ESBL	Extended-Spectrum β-Lactamases
ExPEC	Extraintestinal Pathogenic *E. coli*
FAO	Food and Agriculture Organization, Rome, Italy
GHP	Good Hygiene Practice
GMP	Good Manufacturing Practice
GTA	Gene Transfer Agent
HACCP	Hazard Analysis - Critical Control Point

HGT	Horizontal Gene Transfer
HUS	Hemolytic Uremic Syndrome
ICE	Integrative and Conjugative Element
IFT	Institute of Food Technologists, Chicago, USA
IME	Integrative Mobilizable Element
IPEC	Intestinal Pathogenic *E. coli*
IPTG	Isopropyl-Thio-β-D-Galactoside
IS	Insertion Sequence
IS*CR*	Insertion sequence common regions
LB	Luria Bertani
MAP	Modified Atmosphere Packaging
MI	Mobile Integron
MIC	Minimal Inhibitory Concentration
MLST	Multilocus Sequence Typing
MRD	Maximum Recovery Diluent
MRS	de Man, Rogosa and Sharpe
OD	Optical Density
OIE	Office International des Epizooties (World Organisation for Animal Health), Paris, France
OMV	Outer Membrane Vesicle
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
QPS	Qualified Presumption of Safety
RFLP	Restriction Fragment Length Polymorphism
RTE	Ready-To-Eat
SSC	Side Scatter Light
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks, Brussels, Belgium
SGI1	*Salmonella* genomic island 1
STEC	Shiga toxin-producing E. coli
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TSA	Tryptone Soya Agar
TSAYE	Tryptone Soya Agar with Yeast Extract

TSB	Tryptone Soya Broth
TSBYE	Tryptone Soya Agar with Yeast Extract
UHT	Ultra-High-Temperature
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UTI	Urinary Tract Infection
VBNC	Viable But Non-Culturable
VTEC	verocytotoxin-producing or verocytotoxigenic *E. coli*
WHO	World Health Organization, Geneva, Switzerland

Table of contents

Chapter 1

Introduction

1. Antibiotic resistance

1.1. Introduction

The importance of the discovery and the use of antibiotics for the history of mankind is undeniable. In 1928, the first antibiotic, penicillin, was serendipitously discovered by Sir Alexander Fleming. However, it was not until during World War II that the large scale production was developed (Kardos & Demain, 2011) and it is not unthinkable that the availability of penicillin to the Allies has affected the outcome of this war (Wainwright, 2004). This large scale production meant the onset of the "golden age of antibiotic discovery", which took place between the 1940s and the 1960s, while since the 1970s the discovery of new antibiotic classes has slackened (Walsh & Wencewicz, 2014). Most of the antibiotics which were introduced since that time have been chemical modifications of previously discovered classes (Powers, 2004). In 1945, in an interview with The New York Times, Sir Alexander Fleming cautioned that misuse of penicillin could lead to the appearance of resistant mutant bacteria (Alanis, 2005). Unfortunately, he has been proven right and nowadays, antibiotic resistance is considered as one of the major global public health threats.

1.2. Definitions

Although the subject of this thesis is resistance to "antibiotics", it is nevertheless appropriate to define a number of commonly used related terms. The general term "antimicrobial" refers to any compound, including antibiotics and biocides, that acts in an inhibitory or lethal way against microorganisms (Capita & Alonso-Calleja, 2013). Antibiotics are natural, semi-synthetic or synthetic drugs, which are administered at low concentrations to treat, control or prevent infectious diseases in humans, animals or plants (Capita & Alonso-Calleja, 2013). They are also used as growth promoters in animal production to improve the efficiency of feed utilization. The mechanisms by which antibiotic growth promoters (AGPs) exert their beneficial function is not totally clear yet. The most widely accepted hypothesis is that AGPs modulate the intestinal microbiota by an antibacterial action which can result in: I) decreased competition for nutrients, II) reduction in microbial metabolites that depress growth, III) enhanced nutrient absorption by reduction in gut size, including thinner intestinal villi and total gut wall, and IV) reduction in opportunistic pathogens and subclinical infection (Dibner & Richards, 2005). The antibacterial mechanism of AGPs however has been doubted and an alternative hypothesis, an anti-inflammatory effect of AGPs, has been proposed (Niewold,

2007). According to this investigator, phagocytic inflammatory cells can accumulate antibiotics resulting in an attenuation of the inflammatory response. Consequently, the levels of proinflammatory cytokines would be lower than those in untreated animals, which would result in a lower catabolic stimulus (Niewold, 2007). Biocides on the other hand are defined as "Active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means." (OJEC, 1998).

Antibiotics are subdivided in different classes with similar structure and mode of action. Cross-resistance occurs when the resistance mechanism confers resistance to most or all members of a class, due to the fact that they have the same or similar target and mode of action. However, cross-resistance can also occur among unrelated classes as a consequence of an overlapping target or of the low specificity of the resistance mechanism (EFSA, 2008).

The term "co-resistance" is applied when resistance genes are physically linked to each other. This is the case when the different resistance genes are part of the same genetic element (*e.g.* plasmid, transposon or integron). In co-resistance, the resistance genes are transferred in a single event and are expressed jointly (Capita & Alonso-Calleja, 2013).

There is no standard definition for "multidrug resistance", however, the following definition was recently proposed: "acquired non-susceptibility to at least one agent in three or more antimicrobial categories" (Magiorakos *et al.*, 2012).

A distinction can be made between intrinsic and acquired resistance. Intrinsic resistance is a feature inherent to a bacterial species and every member of this species exhibits this resistance. On the other hand, bacteria can acquire resistance by horizontal gene transfer, by which they receive antibiotic resistance genes from other bacteria, or by mutation. Mechanisms of horizontal gene spread among bacterial strains or species are often considered to be the main mediators of antibiotic resistance (Woodford & Ellington, 2007). Horizontal gene transfer will be discussed later in this Chapter (see section 2 Horizontal gene transfer). However, mutational resistance may have major clinical importance in certain bacterial species, such as *Mycobacterium tuberculosis*, in which resistance to all therapeutic agents (rifampicin, isoniazid, streptomycin, pyrazinamide, ethambutol and fluoroquinolones) is mediated by mutations or when considering resistance to particular antibiotics, such as for example fluoroquinolones (Woodford & Ellington, 2007). Resistance to fluoroquinolones can result from the accumulation of amino acid substitutions in the enzymes DNA gyrase and

DNA topoisomerase IV, with increasing numbers of mutations generally correlating with increasing MICs (Woodford & Ellington, 2007).

1.3. Mechanisms

Bacteria guard themselves against the action of antibiotics by developing resistance. Resistance to a specific antibiotic can be caused by different mechanisms (Figure 1.1). Furthermore, the different types of resistance mechanisms do not work exclusively. For fluoroquinolones and β-lactams, it is known that more than one resistance mechanism can be active in the same bacterial cell (McDermott *et al.*, 2003). Following mechanisms have been described:

I) Antibiotics can be enzymatically modified or degraded before they reach their target site. This strategy is proven very successful by the numerous β-lactamases and aminoglycoside modifying enzymes (Ramirez & Tolmasky, 2010; Smet *et al.*, 2010);

II) A second mechanism is lowering the internal concentration of antibiotics in the bacterial cell a) by efflux pumps or b) by changing the permeability of the cell membrane. Efflux pumps mediate resistance to a wide variety of antibiotics (Poole, 2005). The efflux can be increased by the acquisition of specific genes or by the overexpression of genes coding for present efflux pumps (Poole, 2005). The membrane structure and composition can act as a natural permeability barrier, presenting the most common form of intrinsic resistance, but it can also change as a result of acquired resistance mechanisms (IFT, 2006). For example, resistance to β-lactams and (fluoro)quinolones can be the result of this resistance mechanism (Ruiz, 2003; Poole, 2004; Pagès *et al.*, 2008);

III) Alteration of the target molecule of the antibiotic by mutations can cause a decrease in affinity between the antibiotic and the target molecule. This mechanism can cause resistance to for example rifampicin and quinolones (Ruiz, 2003; Tupin *et al.*, 2010);

IV) Lastly, bacteria can become resistant by following an alternative metabolic pathway. A typical example of this mechanism is the resistance to sulfonamides and trimethoprim (Capita & Alonso-Calleja, 2013).
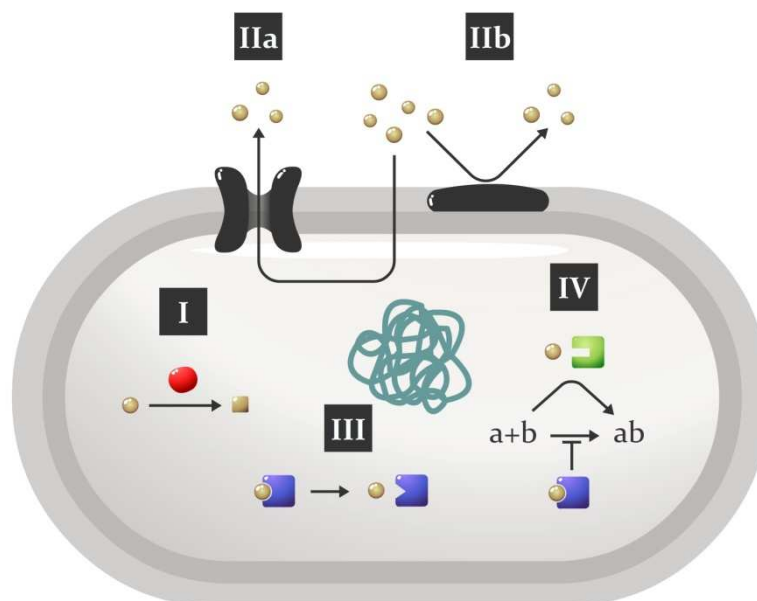
Figure 1.1. *Mechanisms of antibiotic resistance: I) enzymatic modification/degradation of the antibiotic, II) antibiotic concentration decrease by efflux (a) or by changing cell membrane permeability (b), III) target alteration and IV) alternative metabolic pathway.*

1.4. Origin

Although antibiotic resistance poses nowadays an enormous threat to public health, it actually is an ancient natural phenomenon. Genes encoding resistance to β-lactam, tetracycline and glycopeptide antibiotics were found in 30000 years old permafrost sediments (D'Costa *et al.*, 2011). An antibiotic resistance screening of the Lechuguilla cave in New Mexico, which has been isolated for more than 4 million years, revealed the presence of multidrug resistant bacteria (Bhullar *et al.*, 2012). Resistance to 3 - 4 different antibiotic classes was found, on average, in 70% of the Gram-positive strains and in approximately 65% of the Gram-negative strains. Three *Streptomyces* spp. strains were even resistant to 14 antibiotics (Bhullar *et al.*, 2012).

Antibiotic resistance determinants can have a shielding role in natural environments (with low antibiotic selective pressure), although this does not always seem to be the primary function (Martinez, 2009). They can also be involved in metabolic processes as is the case for a chromosomal acetyltransferase in *Providencia stuartii*, which is involved in the acetylation of peptidoglycan (Macinga & Rather, 1999). Another example are multidrug efflux pumps which can also be implicated in detoxification, virulence, homeostasis or signal trafficking in microbial natural ecosystems (reviewed by Martinez *et al.*, 2009). Since the beginning of the

antibiotic era, human activity can have caused a shift in their functionality and has influenced the distribution and abundance of resistance genes (Martinez, 2009; Finley *et al.*, 2013).

1.5. Impact of the use of antibiotics in the food production on antibiotic resistance

During food production, antimicrobial agents are regularly applied in several steps of the production and manufacturing process. This is to obtain at the end food from healthy plants and animals, which has a high hygienic quality and is safe for human consumption. However, these agents can impose a selective pressure on the bacteria which can lead to resistance development. The antimicrobial agents used throughout the food chain include disinfectants, fungicides, antibiotics and feed preservatives in the primary production, disinfectants, food preservatives and decontaminants in the secondary production, disinfectants and food preservatives in the tertiary production and disinfectants at the food consumption stage (Capita & Alonso-Calleja, 2013). The primary production can be divided in animal production (including aquaculture) and plant production. In both domains antibiotics are applied, though the amount of antibiotics used in plant agriculture is small compared to the amounts used in animal production. In 2009 in the United States, for example, the quantity of antibiotics applied to orchards amounted to only 0.12% of the total antibiotics used in animal agriculture (Stockwell & Duffy, 2012). In animal production, the administration of antibiotics serves four goals, namely the treatment, control and prevention of infectious diseases and growth promotion. The European Union uses a more cautious approach ("precautionary principle") than the United States concerning the use of antimicrobials in the food production. This is exemplified by the ban of the use of antibiotics as growth promoters since 2006 in the European Union (OJEU, 2003a). Bacteria that became resistant due to the selective pressure exerted by the administration of antibiotics during primary production can subsequently colonize or infect humans. It has been demonstrated that farm workers have a higher prevalence of resistant gut bacteria compared to the general public or to workers on farms not using antimicrobial growth promoters (Marshall & Levy, 2011). Strong evidence exists that consumers can acquire infections with antibiotic resistant bacteria by the consumption or handling of food that contains antibiotic resistant bacteria (Marshall & Levy, 2011; FAO/OIE/WHO 2003).

1.6. Other factors contributing to antibiotic resistance in food

Antibiotic resistance does not only occur in food as a consequence of the use of antimicrobials throughout the food production chain. During each step of the food production chain contamination with antibiotic resistant bacteria can occur. When contamination occurs after processing, it is called post-contamination. Cross-contamination can occur due to the improper handling of food during processing. In the kitchen a variety of sources for cross-contamination can be present, *e.g.* the work surfaces, towels, the refrigerator and even the presence of household pets (EFSA, 2008).

In some types of food, bacteria are intentionally added. This can have several goals: starter cultures are added for fermentation, probiotics are added for their beneficial effects on the host organism, biopreserving bacteria are added for the extension of shelf life (Verraes *et al.*, 2013). In 2007, EFSA introduced a pre-market safety assessment, Qualified Presumption of Safety (QPS), for the microorganisms that are used in feed/food production in which antibiotic resistance criteria are also included and this list is updated yearly (EFSA, 2007).

Although the application of biocides in the food industry is not explored into more detail in this thesis, it is important to mention that there are indications that the use of these compounds may contribute to the generation of antibiotic resistance (SCENIHR, 2009). Antibiotics and biocides share similarities in their antibacterial properties and in the resistance mechanisms used by bacteria (Davin-Regli & Pagès, 2012). Cross-resistance between biocides and antibiotics can imply efflux pumps or changes in cell envelope, but biofilms can be involved as well (SCENIHR, 2009). Co-resistance also occurs when the genes, encoding the resistant phenotypes, are located together on a single mobile genetic element (Chapman, 2003). Both kinds of resistance have been reported multiple times in association with resistance to quaternary ammonium compounds (Hegstad *et al.*, 2010).

1.7. Significance of the antibiotic resistance problem

The magnitude of the antimicrobial resistance problem worldwide is still largely unknown. The World Health Organization (WHO) has recently published a report in which, for the first time, the current worldwide status of surveillance and information on antimicrobial resistance at country level, in particular antibacterial resistance, was examined (WHO, 2014). Two key findings of this report are: a) very high rates of resistance have been observed in bacteria that cause common health-care associated and community-acquired infections (*e.g.* urinary tract infection, pneumonia) in all WHO regions; b) there are significant gaps in surveillance, and a

lack of standards for methodology, data sharing and coordination, which consequently compromises the ability to assess and monitor the situation. In this report, resistance to third-generation cephalosporins and to fluoroquinolones in *Escherichia coli* was amongst others considered. Pathogenic *E. coli* is the most frequent cause of bloodstream infections, community- and hospital-acquired urinary tract infections, and one of the leading causative agents in foodborne infections worldwide. Third-generation cephalosporins are widely used for intravenous treatment of severe infections in hospitals, while fluoroquinolones are among the most widely used oral antibacterial drugs in the community. For both types of antibiotics, resistance exceeded 50% in five out of the six WHO regions. Both of them are also considered as critically important antimicrobials in the treatment of severe or invasive salmonellosis in humans (EFSA/ECDC, 2014).

On European level, antimicrobial resistance surveillance is assured by European law with for example the listing of antimicrobial resistance as a special health issue in Annex 1 of Commission Decision 2000/96/EC on the communicable diseases to be progressively covered by the Community network under Decision No 2119/98/EC of the European Parliament and of the Council (OJEC, 2000) and Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents, which obliges Member States to monitor and report antimicrobial resistance in *Salmonella* and *Campylobacter* isolates obtained from healthy food-producing animals and from food (OJEU, 2003b). The antibiotic resistance (%) in *Salmonella* spp. from humans and from food and animals for 2012 in the EU are represented in Table 1.1 and 1.2. Concerning the human isolates, the antibiotic resistance (%) is also reported separately for *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Salmonella enterica* subsp. *enterica* serovar Typhimurium.

The European Centre for Disease Prevention and Control (ECDC) publishes annually on the one hand the "Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)" and on the other hand the "European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food", a joint report together with EFSA. EARS-Net is a European wide network of national surveillance systems, providing European reference data on antimicrobial resistance for public health purposes by performing surveillance of antimicrobial resistance based on invasive isolates from blood or cerebrospinal fluid from eight bacterial microorganisms of public health importance, one of which is *E. coli*. For 2012, following EU/EEA population-weighted mean percentages of resistance were reported in *E coli*: 57.4% for aminopenicillins, 11.8% for third-generation cephalosporins, 22.3% for fluoroquinolones, 10.3% for aminoglycosides and < 0.1% for

carbapenem resistance (ECDC, 2013). Statistically significant increases in EU/EEA population-weighted mean percentage of resistance during the time period 2009-2012 was reported for third-generation cephalosporins and aminoglycosides (ECDC, 2013).

Human health consequences related to foodborne antibiotic resistance include (Angulo et al., 2004): I) infections that would not otherwise have occurred if the pathogens were not resistant, which can be expressed as the "attributable fraction". This refers to the increased risk that people, who are treated with antibiotics for whatever reason, have of developing illness with pathogens resistant to the particular antibiotic; II) increased frequency of treatment failure and increased severity of infection. These can lead to prolonged duration of illness, increased frequency of bloodstream infections, increased hospitalization and increased mortality. Antibiotic resistance may cause early empirical treatment to be less efficient and may limit the choices of treatment (Mølbak, 2005). Furthermore, the risk of complications is amplified by treatment failure (Capita & Alonso-Calleja, 2013). Additionally, antibiotic resistance may be associated with increased virulence by co-selection of antibiotic resistance and virulence mechanisms. Several mobile genetic elements such as plasmids, integrative and conjugative elements, and outer membrane vesicles may be involved (Beceiro et al., 2013).

These consequences have mainly been studied in relation to antibiotic resistant *Salmonella* and *Campylobacter* (reviewed by Angulo, 2004; by Tollefson & Karp, 2004 and by Mølbak, 2005). Calculations suggest that in the USA antibiotic resistance annually results in an additional 29379 non-typhoidal *Salmonella* infections, leading to 342 hospitalizations and 12 deaths, and an additional 17668 *Campylobacter jejuni* infections, leading to 95 hospitalizations (Barza & Trevors, 2002). Recently, this was also analyzed for third-generation cephalosporin resistant *E. coli* (de Kraker et al., 2011; Collignon et al., 2013). The burden of disease associated with blood stream infections caused by third-generation cephalosporin resistant *E. coli* in Europe was estimated at 2712 excess deaths and 120065 extra hospital days, based on data from 2007 (de Kraker et al., 2011). Inevitably, this will result in economical consequences. The total costs attributable to excess hospital stays for blood stream infections caused by third-generation cephalosporin resistant *E. coli* were 18.1 million € (de Kraker et al., 2011). In Europe in 2007, the number of infections caused by selected multidrug resistant bacteria (with the focus on bacteria most frequently isolated from blood cultures and with markers for multidrug resistance) was estimated at approximately 400000 with 25000 attributable deaths and 2.5 million extra hospital days (ECDC/EMEA, 2009). The costs associated with these infections, including costs concerning patient care and productivity losses, were estimated at 1.5 billion € (ECDC/EMEA, 2009).

Another aspect to keep in mind is that there is increasing evidence that foodborne infections do not only affect the gastrointestinal tract, but that urinary tract infections (UTI) caused by antibiotic resistant *E. coli* can also have a foodborne origin (in particular poultry) (Nordstrom *et al.*, 2013). This evidence originates from studies demonstrating the genetic relationship between foodborne *E. coli* and *E. coli* from UTI cases and the capability of foodborne *E. coli* to cause UTIs *in vivo* (reviewed by Nordstrom *et al.*, 2013). An epidemiological study furthermore indicated that poultry or pork possibly represent a food reservoir for antimicrobial resistant, UTI-causing *E. coli* (Manges *et al.*, 2007). An overview of extraintestinal pathogenic *E. coli* lineages with a possible food reservoir and those with no known food animal reservoir has recently been given by Manges & Johnson (2012). This consequently enlarges the implications of antibiotic resistant *E. coli* in the food supply.

Table 1.1. *Antimicrobial resistance (%) in* Salmonella *spp. (all non-typhoidal serovars),* S. enterica *serovar Enteritidis and* S. enterica *serovar Typhimurium isolates from humans in 2012, using clinical breakpoints (EFSA/ECDC, 2014).*

	Amp	Cef	Chl	Cip	Gen	Kan	Nal	Str	Sul	Tet	Tri
Salmonella spp.	27.6	1.1	5.7	5.1	5.0	1.7	14.4	23.6	28.9	30.0	6.9
S. enterica serovar Enteritidis	5.7	0.7	0.4	4.9	5.5	0.1	18.8	0.9	1.9	2.5	1.5
S. enterica serovar Typhimurium	66.6	0.9	18.3	2.2	3.0	1.7	6.4	46.2	62.4	63.7	12.0

Amp: ampicillin; Cef: cefotaxime; Chl: chloramphenicol; Cip: ciprofloxacin; Gen: gentamicin; Kan: kanamycin; Nal: nalidixic acid; Str: streptomycin; Sul: sulfonamides; Tet: tetracycline, Tri: trimethoprim.

Table 1.2. *Antimicrobial resistance (%) in* Salmonella *isolates from food and animals in 2012, using harmonised epidemiological cut-off values (EFSA/ECDC, 2014).*

	Amp	Cef	Chl	Cip	Gen	Nal	Sul	Tet
Broilers & spent hens meat	19.9	4.3	5.9	63.1	4.2	57.3	53.0	48.9
Gallus Gallus (fowl)	21.2	4.5	4.4	37.3	4.7	34.3	28.3	25.9
Meat from pigs	47.5	0.9	12.6	7.6	2.4	4.2	53.5	49.2
Pigs	60.2	2.3	14.2	7.6	3.4	5.8	63.3	63.3
Meat from bovine animals	40.0	1.4	9.9	20.0	9.4	8.2	40.8	39.5
Cattle	34.5	0.4	15.5	9.1	1.1	9.1	42.4	36.0

Amp: ampicillin; Cef: cefotaxime; Chl: chloramphenicol; Cip: ciprofloxacin; Gen: gentamicin; Nal: nalidixic acid; Sul: sulfonamides; Tet: tetracycline.

2. Horizontal gene transfer (HGT)

2.1. Introduction

In the dissemination of antibiotic resistance, not only the bacteria play a major role, but transferable antibiotic resistance genes are key players as well. These antibiotic resistance genes can be transferred by means of horizontal gene transfer (HGT). Three main mechanisms can be distinguished in HGT, namely conjugation, transformation and transduction (Figure 1.2). In the case of antibiotic resistance transfer, conjugation is considered the most important mechanism since many antibiotic resistance genes are situated on mobile elements such as plasmids and conjugative transposons. Furthermore, conjugation of broad-host-range plasmids enables DNA to be transferred over genus and species borders, whereas transformation and transduction are usually more limited to the same species (Mathur & Singh, 2005). Conjugative or mobilizable plasmids are the most common transmission vectors for antibiotic resistance genes (Boerlin & Reid-Smith, 2008; Hawkey & Jones, 2009).
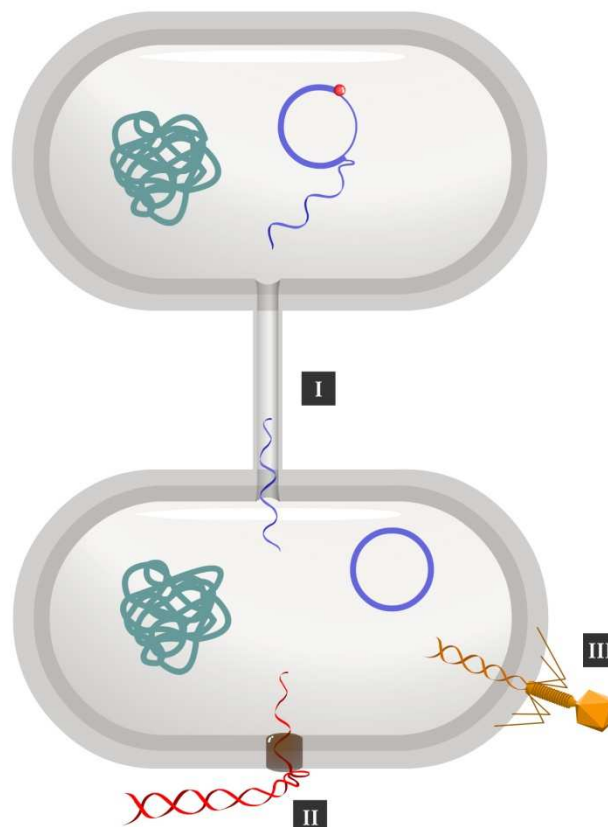
Figure 1.2. *The three main mechanisms of horizontal gene transfer: I) Conjugation (transfer of genetic material from a donor to a recipient bacterium), II) Transformation (uptake of free DNA) and III) Transduction (transfer of genetic material by bacteriophages).*

2.2. *Mechanisms of HGT*

2.2.1. Conjugation

Conjugation involves the transfer of genetic elements from a donor bacterium to a recipient bacterium. This mechanism requires physical contact between the bacteria. A wide range of genetic elements are transferred by means of conjugation of which **plasmids** are the most common. Three characteristics are inherent to plasmids: I) they are able to exist extrachromosomally and replicate autonomously; II) they can transfer between distinct hosts, III) they do not possess housekeeping genes essential to their hosts (Skippington & Ragan, 2011). Another important feature of plasmids is that they can carry resistance genes for practically every type of antibiotic (Barlow, 2009). There are two types of plasmids, namely conjugative plasmids and mobilizable plasmids. The latter needs the help of a conjugative plasmid to be able to transfer to other cells. Mobilizable plasmids are rather small (< 10kb) compared to the conjugative plasmids (> 30kb). This difference in size is explained by the presence of genes encoding conjugation functions in the conjugative plasmids (Bennett, 2008). Plasmids have developed a remarkable diversity of strategies to enable DNA transfer. However, basic conjugative steps can be described by common mechanistic principles (Zechner *et al.*, 2000). The first step is the intimate contact between cells. Gram-positive and Gram-negative bacteria differ in the mechanisms used to achieve cell-cell contact. In Gram-negative bacteria this is promoted by plasmid-determined extracellular filaments, sex pili. An interaction between the tip of the sex pilus and the surface of the recipient cell leads to the initial contact. Intimate association of the cell surfaces is achieved by pilus retraction. Subsequently, a mating bridge between the cells is formed, serving as a conduit for DNA (Zechner *et al.*, 2000). In Gram-positive bacteria cell-cell contact is induced by other mechanisms such as pheromones secreted by the recipient cells or by aggregation (Grohmann *et al.*, 2003). The second step is the transport of the DNA as a single-stranded linear molecule. This involves cleavage of the transferring DNA by relaxase at the transfer of origin (*oriT*), resulting in a nucleoprotein complex (relaxosome) which is transported to the recipient cell by a protein export mechanism (Garcillán-Barcia *et al.*, 2009). The majority of conjugative plasmids applies herefore the type IV secretion system (T4SS) (Goessweiner-Mohr *et al.*, 2013). The DNA is pumped into the recipient cell by the coupling protein T4CP. To be established in the recipient cell the incoming plasmid has to be circularized, which is relaxase-mediated, and the complementary strand has to be synthesized. Conjugative plasmids can exhibit a broad- or a narrow-host-range. For the latter, transfer is restricted generally between a small number of similar bacterial species (Bennett, 2008). Many of the conjugative

plasmids are supplied with broad-host-range properties among Gram-negative species and a small number of these plasmids can also transfer between and replicate in both Gram-negative and Gram-positive bacteria (Schröder & Lanka, 2005). It is not our intention to discuss all the other genetic elements which can be transferred by conjugation thoroughly, but they are briefly mentioned to demonstrate the broad range of opportunities that bacteria have for the dissemination of antibiotic resistance.

Transposons (also called jumping genes) are able to move within and between chromosomes and plasmids. In analogy with plasmids, two types of transposons are the conjugative and mobilizable transposons. Conjugative transposons belong to a larger group of mobile genetic elements, the **integrative and conjugative elements (ICEs)**. ICEs are elements that integrate into and excise from the chromosome, replicate with the chromosome and are transferred by conjugation (Burrus *et al.*, 2002; Burrus & Waldor, 2004).

Similarly, mobilizable transposons are part of the **integrative mobilizable elements (IMEs)**. A well known example of an integrative mobilizable element that can contribute to the spread of antibiotic resistance is the *Salmonella* genomic island 1 (SGI1) (Doublet *et al.*, 2005). Two other transposon types are the unit transposon and the composite transposon. The unit transposon encodes an enzyme involved in excision and integration and contains one or several accessory (*e.g.* resistance) genes in one genetic unit (Roberts *et al.*, 2008). In a composite transposon, the DNA segment is flanked by insertion sequences (IS) (Roberts *et al.*, 2008). The first genes which were recognized as being part of composite transposons were antibiotic resistance genes (Merlin *et al.*, 2000). **Insertion sequences** are small elements, carrying only genes necessary for their transposition, and which are mostly delineated by inverted terminal repeats of 10-40 bp (Mahillon & Chandler, 1998). **Insertion sequence common regions (IS*CR*)** elements are IS*91*-like elements which differ from the classical ISs as they lack the typical repeats at the ends and they typically transpose using a rolling circle replication mechanism (Boerlin & Reid-Smith, 2008). Whereas most IS elements need two flanking copies to mobilize genes, IS*CR* elements can transpose adjacent DNA sequences, mediated by a single copy of the element due to the rolling circle transposition (Toleman *et al.*, 2006). IS*CR* elements are remarkable for their close association with a wide variety of antibiotic resistance genes and can contribute to the mobilization of virtually every class of antibiotic resistance genes, including those encoding extended-spectrum β-lactamases (ESBLs), carbapenemases, and enzymes conferring broad-spectrum aminoglycoside resistance, florfenicol/chloramphenicol resistance, and resistance to trimethoprim and quinolones (reviewed by Toleman *et al.*, 2006).

Integrons represent an example of the fascinating ways bacteria evolve to overcome the threat that antibiotics impose. At the beginning of the antibiotic era multiresistance was not anticipated, because the co-appearance of multiple mutations conferring resistance was considered to be beyond the evolutionary potential of a given bacterial population. In the 1950s the first multiresistant bacteria were observed and it soon became clear that the resistance could be transferred (Watanabe, 1963). In the 1970s, multidrug resistance was determined in many cases to be associated with transmissible plasmids (Mazel, 2006). Integrons were first described in the late 1980s (Stokes & Hall, 1989). It is becoming clear that integrons are actually a common component of bacterial genomes with a long evolutionary history and that antibiotic use selected particular integrons from among the environmental pool, resulting in the presence of integrons carrying resistance genes in the majority of Gram-negative pathogens (Gillings, 2014). The ongoing use of antibiotics in clinical and agricultural practice has made mobile resistance integrons extraordinarily abundant, in particular class 1 integrons. In healthy humans, including infants who have not yet been exposed to antibiotics, they have been observed in 10 to 50% of commensal bacteria. The integron carriage by commensal *E. coli* in farm animals can rise to 80% (Gillings, 2014).

An integron is an immobile element which can capture, integrate and express or release gene cassettes. All the elements necessary for the integration and expression or excision of the gene cassette(s) are located within the 5'-CS region, namely an *intI* gene encoding a site-specific tyrosine recombinase, which catalyzes the specific excision and integration of the gene cassette(s), a recombination site *attI* and a common promoter, Pc, for the expression of the genes. A gene cassette consists of a gene and a recombination site, *attC*, by which the cassette can be integrated in the integron by site-specific recombination. Figure 1.3 shows how gene cassettes are integrated in an integron. Integrons can be associated with mobile DNA elements such as plasmids and transposons (mobile integrons, MI) or they can be associated with the bacterial chromosome (chromosomal integrons, CI) (Cambray *et al.*, 2010). Chromosomal and mobile integrons differ in the number and the function of gene cassettes. Chromosomal integrons can carry a variable number of gene cassettes, ranging from zero to hundreds, which are usually not implicated in antimicrobial resistance, while MI contain a limited number of gene cassettes, mostly involved in antibiotic resistance (Domingues *et al.*, 2012). Five different integron classes can be distinguished among the mobile integrons, however only the first three classes are historically associated with the dissemination of multiresistance (Cambray *et al.*, 2010). There appears to be a link between integrons and multiresistance.

Nagachinta and Chen (2009) reported that all integron positive Shiga toxin-producing *E. coli* (STEC) strains examined were resistant to at least three different antibiotics.
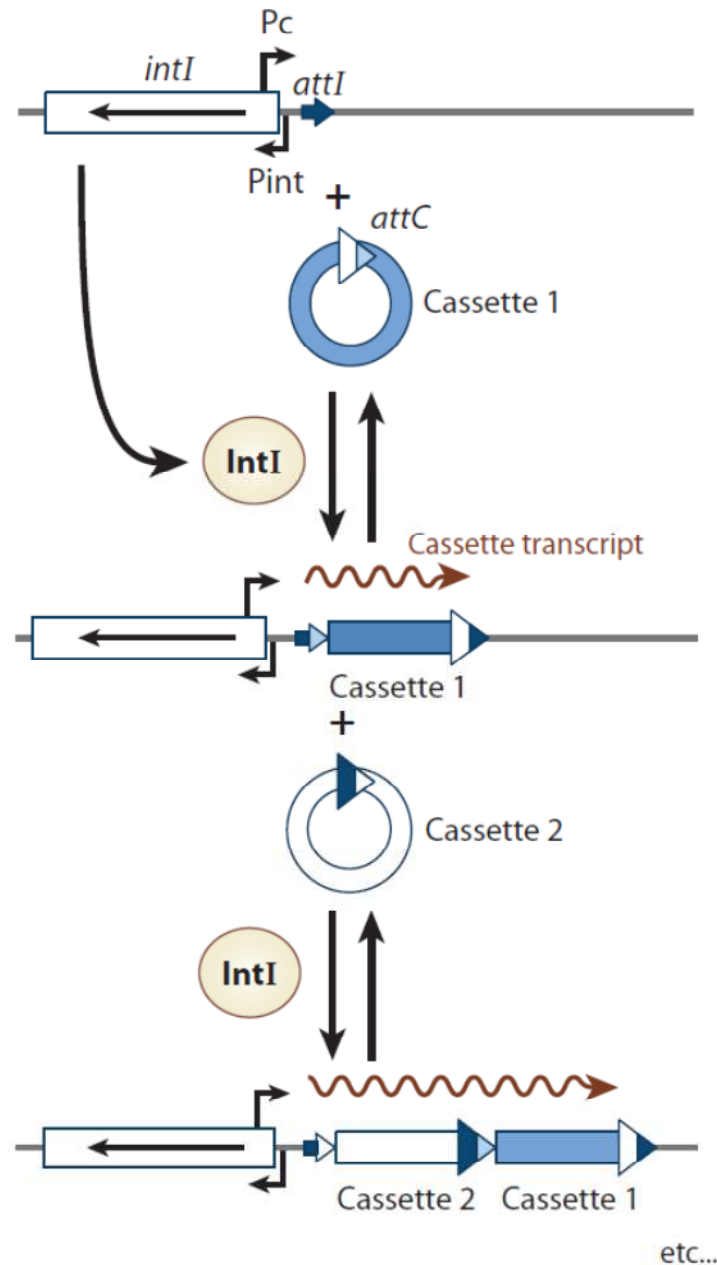
***Figure 1.3.** Integration of gene cassettes in an integron. The integrase (IntI) catalyses the site-specific recombination between the* attI *of the integron and the* attC *of the gene cassette. The integrated gene cassettes are subsequently expressed with the help of the Pc promoter. (Source: Cambray* et al.*, 2010)*

2.2.2. <u>Transformation</u>

Transformation involves the successful uptake of free DNA from the environment. During this process several steps can be distinguished. The first step is the release of DNA from cells. Competent cells can subsequently take up this free DNA. The next step is the stable integration of the DNA in the recipient cell. The last step in a successful transformation is the expression of the acquired trait (Lorenz & Wackernagel, 1994). DNA can be released by bacteria both passively after cell death or actively by secretion (Nielsen *et al.*, 2007). This extracellular DNA has to circumvent enzymatic degradation and chemical or physical inactivation in the environment. The matrix surrounding the DNA influences the stability of DNA. This has been demonstrated for example in thermally treated fermented sausages where recombinant DNA was protected against the activity of DNase (Straub *et al.*, 1999). Another example is soil, in which binding to mineral and humic substances protects the DNA from extracellular, microbial DNases and nucleases (Levy-Booth *et al.*, 2007). To be able to undergo natural transformation, bacteria have to develop competence. Natural competence has been described for several bacterial species belonging to different phyla, however, it is expected that more species are able to undergo natural transformation under adequate conditions (de Vries & Wackernagel, 2004; Johnsborg *et al.*, 2007). Not all strains of a species show the same level of transformability (de Vries & Wackernagel, 2004). Furthermore, there is a species dependence of the time span in which competence is developed. For example, bacteria such as *Helicobacter pylori* or *Neisseria* spp. are constitutively competent while in other bacteria natural competence is induced in response of environmental signals (Seitz & Blokesch, 2013). Some bacterial species have a preference for DNA from the same or closely related species, but most natural competent bacteria are not selective (Lorenz & Wackernagel, 1994; Bakkali, 2013). In the case of *Haemophilus influenzae*, a recognition sequence of 11 bp is involved in this specificity, although at low pH heterologous DNA can bind, suggesting that non-specific DNA uptake can also occur (Lorenz & Wackernagel, 1994). Gram-positive and Gram-negative bacteria have similar DNA uptake mechanisms with differences inherent to the differences in the cell wall structure of Gram-positive and Gram-negative bacteria (Dubneau, 1999; Chen *et al.*, 2005). Under laboratory conditions, competence can be induced by different methods in a wide range of bacteria (Aune & Aachmann, 2010). Integration in the bacterial genome is necessary for the persistence of the internalized chromosomal DNA. This can be achieved by homologous recombination, illegitimate recombination and homology facilitated illegitimate recombination (de Vries & Wackernagel, 2004).

Species in which natural transformation has contributed to antibiotic resistance are for example streptococci, *Neisseria meningitidis* and the foodborne pathogen *C. jejuni* (Bowler *et al.*, 1994; Janoir *et al.*, 1999; Jeon *et al.*, 2008).

2.2.3. Transduction

In transduction, genetic material is transferred by bacteriophages (bacterial viruses).

Three types of genetic exchange are mediated by bacteriophages: generalized transduction, specialized transduction and lysogenic conversion (Brabban *et al.*, 2005). In generalized transduction, virtually any gene from the infected cell can be transferred to a recipient cell. During a lytic infection, the host genome is degraded and bacteriophage reproduction begins. Subsequently, functional virions are generated, however, sometimes bacterial host DNA instead of viral DNA is accidentally packaged by the bacteriophages head assembly system, yielding transducing particles. These transducing particles cannot initiate a normal infection, but they can transfer their DNA to a recipient cell. After entry, this DNA is degraded or recombined with the new host's DNA. A much more efficient mechanism is specialized transduction. Temperate bacteriophages can enter lysogeny after entry, meaning that the expression of bacteriophage genes leading to cell lysis is prevented, and its genome becomes integrated into the bacterial genome (prophage). This generally confers immunity to the host cell against further infection by the same or similar bacteriophages. During the lytic cycle, the prophage is excised. However, sometimes this excision is inaccurate and an adjacent section of the bacterial host's genome is co-excised, which can subsequently be transferred to new hosts. No recipient cells are involved in lysogenic conversion. Lysogenic conversion implies an altering of the phenotype of the infected host by determinants encoded by the prophage of a temperate bacteriophage.

The contribution of this phenomenon to the dissemination of antibiotic resistance has scientifically received less attention than conjugation and transformation. The role of transduction in the dissemination of antibiotic resistance has been reviewed by Brabban *et al.* (2005) with emphasis on its role in *S. enterica serovar* Typhimurium and *Pseudomonas aeruginosa*. Recently, more and more studies have demonstrated the potential of transduction in antibiotic resistance transfer (*e.g.* Zhang & Lejeune, 2008; Di Luca *et al.*, 2010; Varga *et al.*, 2012; Goh *et al.*, 2013).

2.2.4. Other mechanisms of HGT

Next to the three main mechanisms discussed above, other mechanisms of horizontal gene transfer can occur between bacteria, such as vesicle-mediated translocation and gene transfer agents (Keese, 2008).

In Gram-negative bacteria, outer membrane vesicles (OMV) are naturally occurring structures derived from the outer membrane, which were first discovered in the 1960s (Kulp & Kuehn, 2010). More recently, membrane vesicles were also described in Gram-positive bacteria and in Archaea (Manning & Kuehn, 2013). Next to the vesicle-mediated transformation, which is assumed to happen by fusion and consequent transfer of the DNA from the vesicle lumen to the host cell, (outer) membrane vesicles are also involved in *e.g.* virulence, stress response to both internal as external stresses, cross-species interaction, biofilm formation and maintenance (reviewed by Manning & Kuehn, 2013). Vesicle-associated DNA has been found to be both bound to the OMV surface and packaged inside the vesicles. The mechanism by which DNA is packaged as vesicle cargo is not yet clear. Rumbo *et al.* (2011) have proposed two possible mechanisms to explain the presence of DNA inside OMVs: I) plasmids migrate in some way to the periplasm, where they are trapped in OMVs; II) some OMVs could contain both inner and outer membrane compounds, trapping cytoplasmic compounds and even plasmids. The transfer of carbapenem resistance genes by OMVs has been demonstrated in *Acinetobacter baumannii* strains (Rumbo *et al.*, 2011).

Gene transfer agents (GTAs) are phage-like elements with tailed-phage structures that package small segments of the genome of a GTA-producing cell and transmit these genes throughout the environment (Lang *et al.*, 2012). They differ at several points from phages: I) the production of GTAs is not the result of a phage infection. Rather, the encoding genes are contained within the genome of the cell that produces the GTAs; II) the amount of DNA that it contains is a random piece of the genome of the producing cell and is insufficient to encode the protein components of the particle itself, while in generalized transducing phages, the fragments of packaged DNA are the size of the phage genome and usually only an occasional particle contains host genes (Lang *et al.*, 2012). GTAs are presumably released into the environment by lysis of the producing cell after which it is likely that GTAs bind to recipient cells via specific tail–receptor interactions, but the receptor has not been identified yet (Lang *et al.*, 2012). Four genetically unrelated GTAs have been identified to date, but a lot more GTA like elements seem to exist (Lang *et al.*, 2012). The GTA of *Brachyspira hyodysenteriae*, VSH-1, was able to transfer tylosin and chloramphenicol resistance genes between *Brachyspira hyodysenteriae* strains after antibiotic induction (Stanton *et al.*, 2008).

2.3. Effect of food matrices and food processing on HGT

A lack of knowledge exists concerning the significance of antibiotic resistance gene transfer in food products, neither is there a lot of information about the extent to which food processing contributes to the occurrence of HGT.

Successful plasmid transfer by conjugation was demonstrated for example between *Lactobacillus curvatus* strains during sausage fermentation (Vogel *et al.*, 1992), between *Enterococcus faecalis* strains during cheese and sausage fermentations (Cocconcelli *et al.*, 2003), between *Bacillus thuringiensis* and *Bacillus cereus* in milk and dairy products (Van der Auwera *et al.*, 2007; Modrie *et al.*, 2010), from *S. enterica serovar* Typhimurium to *E. coli* in milk and ground meat (Walsh *et al.*, 2008), between *Lactococcus lactis* strains in yoghurt (Toomey *et al.*, 2009a), from *E. faecalis* to bacteria involved in meat fermentation during sausage fermentation (Gazzola *et al.*, 2012) and between *Listeria monocytogenes* strains on salmon and cheese (Bertsch *et al.*, 2013). Kruse & Sørum (1994) investigated the transfer of R plasmids in minced meat (between *E. coli*) and in fish (from *Aeromonas salmonicida* subsp. *salmonicida* to *E. coli*) on a cutting board to simulate food processing in the kitchen. Conjugation was found to occur in both food products as well as on the wooden cutting board in the case of minced meat. However, lower bacterial numbers were found on the cutting board which was possibly due to the bactericidal properties of wood.

It has been shown that sublethal stresses in modern food preservation systems, such as temperature, reduced pH, increased osmotic stress, can have an increasing effect on conjugation rates. Concerning temperature, there is a general consensus that low temperatures have a negative effect on plasmid transfer (Fernandez-Astorga *et al.*, 1992). Walsh *et al.* (2008) studied the effect of low temperature on antibiotic resistance transfer from *S. enterica* serovar Typhimurium to *E. coli* in LB broth, milk and ground meat. Transfer was detected in all three media at 25 and 37 °C, whereas transfer at 15 °C was only observed in ground meat. No transfer was observed at 4 °C, which might be explained by the overall reduction in the metabolic rates of the mesophilic donor and recipient strains used (Walsh *et al.*, 2008). Fernandez-Astorga *et al.* (1992) observed plasmid transfer between *E. coli* in a temperature range between 8 and 37 °C, both in Tryptone Soya Broth (TSB) as in ultrapure distilled water. Although *E. coli* belongs to the mesophilic bacteria, which normally have minimal growth temperatures between 10 and 15 °C, this bacterium is still able to grow at temperatures $\geq$ 7 °C (Jones *et al.*, 2004). Varying the pH (6.0 – 8.5) had no effect in TSB nor in ultrapure distilled water (Fernandez-Astorga *et al.*, 1992). A study on the effect of pH on antibiotic resistance transfer between *Lactococcus lactis* strains demonstrated that varying

the pH of the transfer medium between pH 6.0 and 7.0 had no significant effect on transfer rate, while at pH 8.0 transfer was inhibited (Toomey *et al.*, 2009b). Together with previous studies, this study indicates that the optimum pH may depend on the nature of the plasmid, the physiology of donor and recipient cells, or various combinations of these and perhaps other complex factors (Toomey *et al.*, 2009b). High salt stress (5% NaCl) increases plasmid transfer frequency in *B. thuringiensis* (Beuls *et al.*, 2012). A stimulating effect of the previously mentioned stress factors was observed when prestressed inocula were used (Mc Mahon *et al.*, 2007a).

Concerning transformation, the research performed in food matrices has been focused rather on the effect of food processing on transformation in general than on transformation of antibiotic resistance genes. Bauer *et al.* (1999) demonstrated that transformation of *E. coli* took place in a variety of foodstuffs, even at low temperatures and under conditions mimicking homogenization of milk. Bräutigam *et al.* (1997) found a strong decrease in transformation frequency when chromosomal DNA was pre-incubated in ultra-high temperature (UHT) milk at 20 °C, while the decrease in transformation frequency happened slower when the pre-incubation temperature was 8 °C. van den Eede *et al.* (2004) provide a summary of food chain related factors that can affect DNA integrity. The effect of processing on DNA degradation has been investigated in a variety of food products such as sugar beets, fermented sausages, potato products, orange juice, soy milk, tofu,… (Klein *et al.*, 1998; Straub *et al.*, 1999; Bauer *et al.*, 2003; Kharazmi *et al.*, 2003; Bauer *et al.*, 2004; Weiss *et al.*, 2007). The food matrix may constitute a protective environment for DNA. Kharazmi *et al.* (2003) analyzed the DNA degrading factors during production of soymilk, tofu, corn masa, and cooked potatoes. For soymilk and tofu, grounding was the most important degrading factor, while further treatment, which was boiling in the case of soymilk, did not have an additional effect. This indicates that a specific food matrix may have a protective effect to certain degrading factors, as boiling of the potatoes strongly degraded DNA. This was also seen in thermally treated fermented sausages (Straub *et al.*, 1999).

The effect of food matrices and food processing on transduction has been studied to a lesser extent. Studies analyzing transduction of Stx phages in milk, bottled water, orange juice, salad and ground beef demonstrated that transduction did not take place at low pH or at low temperature (Imamovic *et al.*, 2009; Picozzi *et al.*, 2012). On the other hand, Aertsen *et*

al. (2005) demonstrated that high hydrostatic pressure could induce Stx prophages in *E. coli* in LB medium and in whole milk.

3. Food production and preservation: Bacteria, biofilm, food processing

3.1. Introduction

Early 19th century, the initial steps in food microbiology were taken by Louis Pasteur, who was the first to acknowledge the presence and role of microorganisms in food by demonstrating that the souring of milk was caused by microorganisms (Jay *et al.*, 2005). At the end of the same century *Salmonella* was isolated for the first time from a food poisoning outbreak (Griffith, 2006). Bacteria present in food have several roles. They can be beneficial for the food production process (starter cultures and biopreservatives) or for human health (probiotics), but they can also cause spoilage of food or be pathogenic.

The food industry strives to I) avoid and eliminate contamination with spoilage or pathogenic microorganisms, and II) minimize or inhibit microbial growth during processing and storage. Several techniques applied in the food industry are based on affecting intrinsic, extrinsic and implicit factors. Intrinsic factors are parameters which are inherent to the food product such as pH, water activity (a_w), redox potential, nutrient content (water, energy source, nitrogen source, minerals, vitamins), natural antimicrobial components (*e.g.* lysozyme in egg white) and biological structures (*e.g.* skins of fishes and carcasses, peels of vegetables and fruits, …). Extrinsic factors include parameters of the surrounding environment, namely temperature, relative humidity and atmosphere composition. The implicit factors comprise the mutual interactions among the members of the microbial community. To avoid contamination the food industry applies hygienic programs based on Hazard Analysis - Critical Control Point (HACCP) evaluation. HACCP can be defined as a methodology that identifies, evaluates, and controls hazards that are significant for food safety (Jacxsens *et al.*, 2009).

In this thesis, a limited number of bacterial species was used. They are a model for some of the roles bacteria may have in food. The extrinsic factors temperature and modified atmosphere were explored, as these can be considered as the primary extrinsic factors influencing microbial growth (Montville & Matthews, 2007).

3.2. Bacteria

In this work, three important foodborne pathogens were used as model organisms in the experiments, namely *Salmonella* spp., human pathogenic *E. coli* and *L. monocytogenes*. Based on a Belgian study in which foodborne zoonoses were prioritized, these pathogens were classified in the "most important" group (Cardoen *et al.*, 2009). Furthermore, *Pseudomonas*

putida and *Lactobacillus sakei* subsp. *sakei* were used as model donor organisms, representing other roles that bacteria present in food can have.

3.2.1. *Salmonella* spp.

The genus *Salmonella* comprises two species, *Salmonella enterica* and *Salmonella bongori*, which together contain more than 2500 serovars. The species *S. enterica* is divided in 6 subspecies. The subspecies *S. enterica* subsp. *enterica* is involved in the vast majority of the *Salmonella* infections in mammals and birds. Strains of the other subspecies only sporadically cause infections and are considered commensals of cold-blooded vertebrates (Katribe *et al.*, 2009). The same is true for *S. bongori* (Giammanco *et al.*, 2002). It is already known for a long time that *Salmonella* is a causative agent for foodborne disease. In 1880, it was found the causative agent of a food poisoning outbreak (Griffith, 2006). In 2005, reporting of foodborne outbreaks became mandatory in the EU (EFSA, 2006). In the time period 2005-2011, *Salmonella* was the most frequent causative agent of foodborne outbreaks in the EU. Figure 1.4 shows an overview for the time period 2007-2011 of the number of confirmed human salmonellosis cases, case fatality rates, number of verified or strong evidence foodborne outbreaks and the food implicated in these outbreaks. Eggs and derived products are responsible for the majority of the *Salmonella* associated foodborne outbreaks in Europe (Figure 1.4). The case fatality related to *Salmonella* infections is low, staying far below 1%. Clinical symptoms caused by *Salmonella* are amongst others nausea, vomiting, fever, chills, abdominal pain, myalgias, arthralgias and headache (Sánchez-Vargas *et al.*, 2011). Usually the symptoms are mild in nature and the *Salmonella* infection is self-limiting. However, sometimes more serious conditions appear and consequently effective antibiotic treatment is crucial. Obviously, antibiotic resistance can jeopardize these antibiotic treatments. In *Salmonella*, the degree of antibiotic resistance depends on the serotype (Su *et al.*, 2004). For example, *S. enterica* serovar Enteritidis is a rather susceptible serotype, while *S. enterica* serovar Typhimurium is rather a resistant serotype (Su *et al.*, 2004). *Salmonella* acquires resistance genes primarily via plasmids and class 1 integrons (Alcaine *et al.*, 2007). Michael *et al.* (2006) gives a comprehensive overview of the resistance genes, which were detected in *Salmonella*, their mode of action and their location in the bacterial genome.
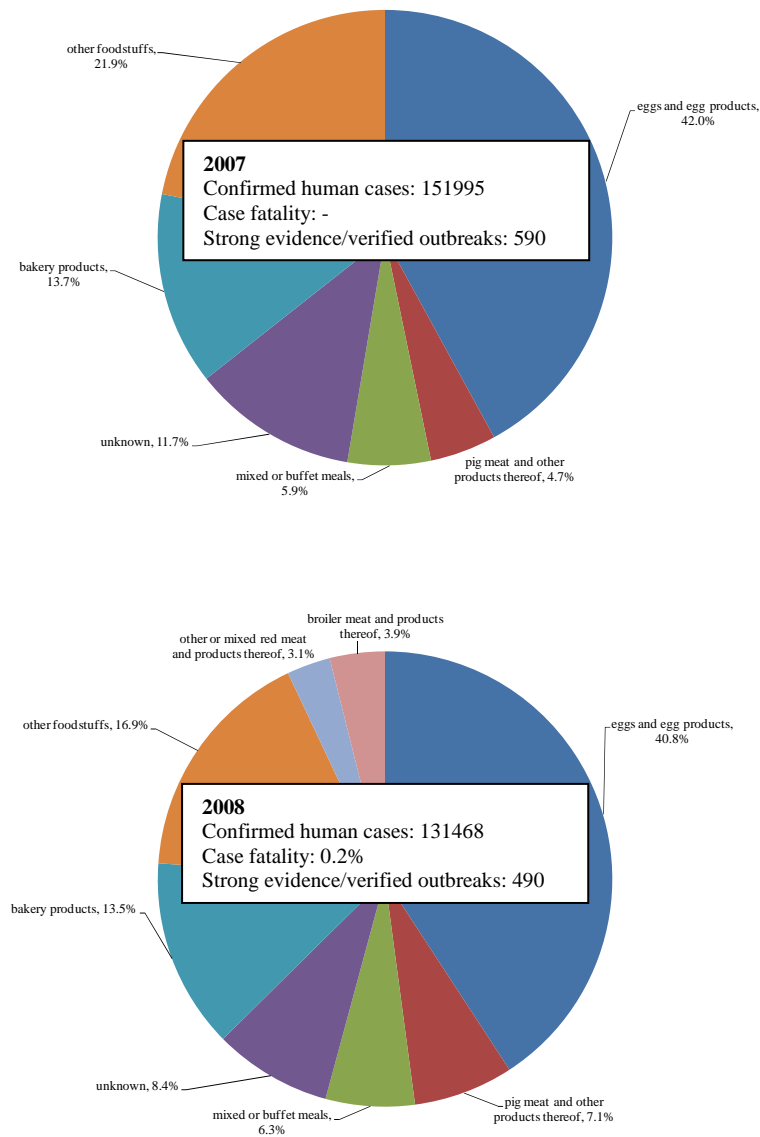
Figure 1.4. Salmonella *spp.: Overview of the number of confirmed human cases, the case fatality rates, the number of verified or strong evidence foodborne outbreaks and distribution of food implicated in these outbreaks (2007-2011) (EFSA/ECDC 2009a, 2009b, 2010, 2011, 2012, 2013).*

2009
Confirmed human cases: 108614
Case fatality: 0.08%
Strong evidence/verified outbreaks: 324

eggs and egg products, 49.1%

bovine meat and products thereof, 3.1%

broiler meat and products thereof, 5.2%

other foodstuffs, 13.9%

bakery products, 10.2%

unknown, 9.3%

mixed or buffet meals, 5.6%

pig meat and other products thereof, 3.7%

2010
Confirmed human cases: 99020
Case fatality: 0.13%
Strong evidence/verified outbreaks: 341

eggs and egg products, 43.7%

other or mixed meat and products thereof, 4.1%

bovine meat and products thereof, 4.7%

broiler meat and products thereof, 5.3%

other foodstuffs, 10.6%

bakery products, 14.4%

mixed or buffet meals, 12.3%

pig meat and other products thereof, 5.3%

2011
Confirmed human cases: 95548
Case fatality: 0.12%
Strong evidence/verified outbreaks: 283

eggs and egg products, 50.5%

vegetables and juices and other products thereof, 2.8%

sweets and chocolate, 6.7%

dairy products (other than cheese), 2.1%

other or mixed meat and products thereof, 4.2%

bovine meat and products thereof, 2.8%

broiler meat and products thereof, 3.2%

other foodstuffs, 8.8%

bakery products, 4.2%

mixed or buffet meals, 9.9%

pig meat and other products thereof, 4.6%

Figure 1.4. Continued.

3.2.2. *Escherichia coli* with focus on Shiga toxin producing *E. coli*

Although *E. coli* is a known commensal inhabiting the intestinal tract of humans and animals, pathogenic variants do occur. A distinction can be made between diarrheagenic or intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC). Based on their pathogenic features, 6 pathotypes can be distinguished among the intestinal pathogenic *E. coli*, namely enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro & Kaper, 1998). The extraintestinal pathogenic *E. coli* causes for example neonatal meningitis, urinary tract infection, sepsis, pneumonia and surgical site infections (Smith *et al.*, 2007). We will focus in this thesis on the Shiga toxin-producing *E. coli* (STEC), a heterogenous subgroup of *E. coli*, which have the production of Shiga toxin, also called verocytotoxin, in common. STEC (*E. coli* O157:H7) was first associated with a foodborne outbreak in 1982 (Riley *et al.*, 1983; Wells *et al.*, 1983). Although more than 380 different STEC serotypes have been isolated from humans with gastrointestinal disease, the majority of human cases appear to be associated with only a limited number of STEC serotypes (Karmali *et al.*, 2010). Since 2008, the number of reported human STEC infections has been increasing in the EU (EFSA/ECDC, 2013). In 2011, there was an extensive outbreak of STEC O104:H4 in Germany which also affected 14 EU member states and the United States (EFSA/ECDC, 2013). This outbreak was associated with the consumption of sprouts (Buchholz *et al.*, 2011). The causative agent in this outbreak was an *E. coli* O104:H4 strain which was extremely virulent. In 22% of the patients haemolytic uremic syndrome (HUS) developed (Frank *et al.*, 2011). On European level, the percentage of HUS ranged between 2.5 – 6.8% in the time period 2006-2010 (EFSA/ECDC, 2007, 2009a; 2010, 2011, 2012). The German *E. coli* O104:H4 strain combined the virulence properties of EAEC and STEC (Frank *et al.*, 2011). This outbreak demonstrates the ease by which foodborne pathogens become mo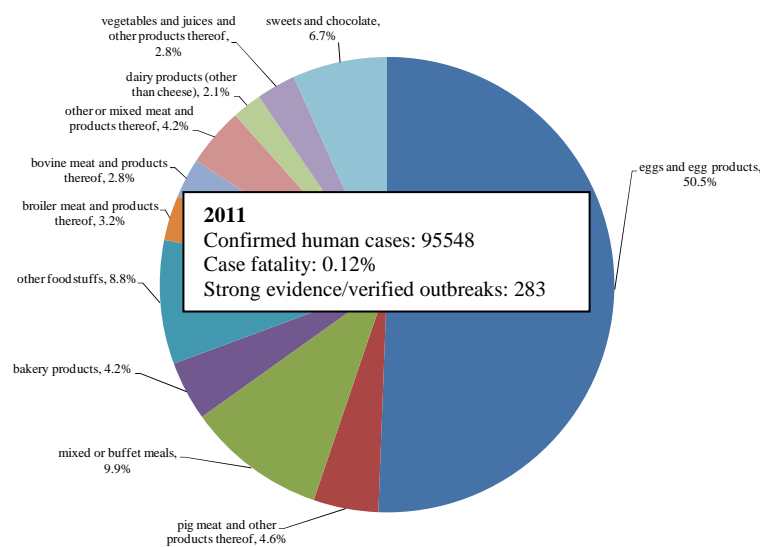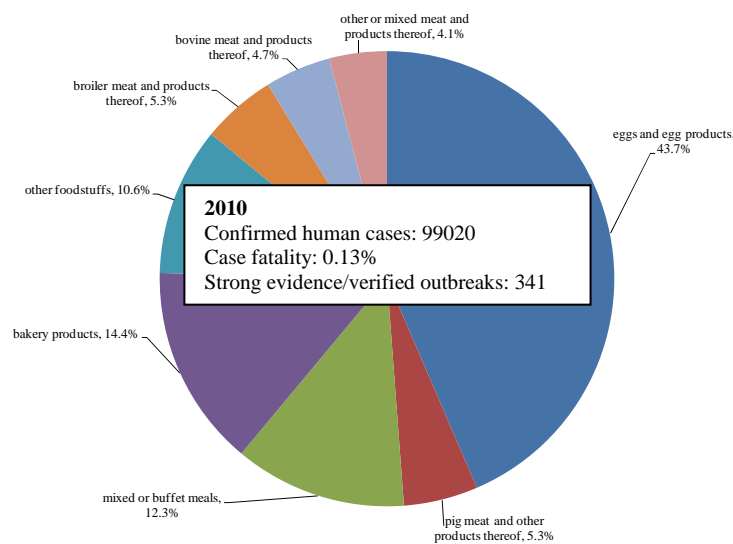re virulent and can subsequently pose a greater threat to the community. Antibiotic resistance in Shiga-toxin producing *E. coli* has not received much attention. A reason for this could be the controversy about antibiotic treatment of STEC infections (Hilbert *et al.*, 2012). It has been demonstrated that certain antibiotics, such as for example fluoroquinolones and trimethoprim-sulfamethoxazole, can increase the risk of developing HUS by inducing Stx production (McGannon *et al.*, 2010). An association between β-lactam antibiotic treatment of O157 infection and the subsequent development of HUS has also been shown (Smith *et al.*, 2012). Nevertheless, it has been reported that antibiotic resistance in STEC is increasing since the early 1990s (although to a lesser extent

compared to the dramatic increase in antibiotic resistance in *S. enterica* and *Campylobacter* spp.) (Threlfall *et al.*, 2000). Buvens *et al.* (2010) demonstrated, by screening a Belgian STEC collection, that both O157 and non-O157 strains are frequently resistant to ampicillin, streptomycin, sulfonamide, and tetracycline. Furthermore, non-O157 strains were significantly more resistant to the previous mentioned antibiotics and to nalidixic acid, kanamycin, chloramphenicol and trimethoprim. The most important elements for resistance carriage and transfer in *E. coli* are antimicrobial resistance plasmids (Hilbert *et al.*, 2012). Recently, the presence of a complex antibiotic resistance locus encoding resistance to six antibiotics (trimethoprim, streptomycin, sulfathiozole, kanamycin, neomycin, β-lactams) on the virulence plasmid of an EHEC O26:H⁻ strain was reported (Venturini *et al.*, 2010). This highlights the danger that antibiotic use can co-select for virulence determinants, which can subsequently lead to an increased disease potential.

3.2.3. *Listeria monocytogenes*

In the bacterial genus *Listeria* 10 species can be discerned (http://www.bacterio.net/listeria.html). The vast majority of the human *Listeria* infections are caused by *L. monocytogenes*, although *Listeria ivanovii*, *Listeria grayi*, *Listeria innocua* and *Listeria seeligeri* have sporadically also been implicated in human infections (Rocourt *et al.*,1986; Perrin *et al.*, 2003; Guillet *et al.*, 2010; Salimnia *et al.*, 2010). *L. monocytogenes* was first identified as a human foodborne pathogen in 1981, causing an outbreak associated with contaminated coleslaw in Canada (Schlech *et al.*, 1983). Although *L. monocytogenes* is a relatively rare foodborne pathogen, it is of significant concern because of the severity of disease it can cause. Where it causes, generally speaking, self-limited gastroenteritis in immunocompetent persons, more severe conditions are encountered in immunocompromised people. In invasive listeriosis typically bacteremia with or without an evident focus of infection is observed, while in pregnant women complications can include spontaneous abortion, stillbirth, preterm delivery and neonatal infection (Drevets & Bronze, 2008). *Listeria monocytogenes* infections are also associated with high case fatality rates. On European level, the case fatality ranged between 12.7 and 20.5% during the time period 2007-2011 (EFSA/ECDC 2009a, 2010, 2011, 2012, 2013).

Refrigerated ready-to-eat (RTE) food products are particularly of concern as they have general physicochemical characteristics that permit *L. monocytogenes* to grow, and their storage for extended times under cold temperature allows the psychrotolerant *L. monocytogenes* to grow, while growth of many competing microorganisms is inhibited

(Chan & Wiedmann, 2009). An aspect that contributes to the contamination of RTE food is the fact that *L. monocytogenes* may be persistently present in a food processing environment for months or years (Tompkin, 2002). In clinical *L. monocytogenes* strains multidrug resistance is seldom observed and acquired resistance is a recent phenomenon (Morvan *et al.*, 2010). While clinical isolates appear to remain susceptible to clinically relevant antibiotics at this time, the number of reports concerning resistance to an expanding spectrum of clinically relevant antimicrobial agents in strains isolated from the food chain are increasing (Allen *et al.*, submitted). The antibiotic resistance mechanisms observed in *L. monocytogenes* involve the acquisition of plasmids and conjugative transposons by means of conjugation and the presence of efflux pumps (Lungu *et al.*, 2011; Allen *et al.*, submitted).

3.2.4. *Pseudomonas putida*

Pseudomonas putida typically inhabits soil and water. In the food industry, this species can be associated with spoilage of meat, milk or fish (Whitfield *et al.*, 2000; Boulares *et al.*, 2013; Doulgeraki & Nychas, 2013). Human infections of *P. putida* are mostly acquired nosocomially (reviewed by Carpenter *et al.*, 2008). Molina *et al.* (2014) have recently characterized the antibiotic resistant determinants of an exceptional multidrug resistant clinical strain, which was resistant to 28 of the 31 tested antibiotics. The strain was resistant to 4 fluoroquinolones, 5 aminoglycosides, 9 β-lactams, 2 polymyxins, nalidixic acid, erythromycin, tetracycline, trimethoprim, chloramphenicol, sulfonamide, vancomycin, esperamicin and susceptible to amikacin, rifampicin and nitrofurantoin (Molina *et al.*, 2014). The antibiotic resistant determinants were located on the chromosome as well as on a plasmid. Resistance to quinolones/fluoroquinolones and cationic antimicrobial peptides was encoded chromosomally, while resistance to aminoglycosides, tetracyclines, β-lactams, chloramphenicol was encoded both chromosomally and on the plasmid. The plasmid also contained resistance determinants to sulfonamides (Molina *et al.*, 2014). Although the plasmid was not conjugative nor mobilizable recombination events with the helper plasmid were observed. Sequencing data indicate that this strain has been in contact and has exchanged DNA with environmental and clinically relevant bacteria.

3.2.5. *Lactobacillus sakei*

Although *Lb. sakei* can be involved in spoilage, it is in general considered to be beneficial due to its role in meat fermentation and meat preservation. The spoilage potential of this species has been demonstrated for example with brined shrimps and sliced cooked ham where it was

associated with contamination during processing (Samelis *et al.*, 1998; Mejlholm *et al.*, 2008, 2012). Spoilage of refrigerated meat products by lactic acid bacteria can be associated with off-odors and off-flavors, discoloration, gas production, slime production and a decrease in pH (Borch *et al.*, 1996). Although fermentation contributes to the unique taste, aroma or texture of certain food products, this was not its original purpose. Fermentation was initially executed for preservation of food (Caplice & Fitzgerald, 1999). Biopreservation serves the extension of shelf life and improvement of food safety by using microorganisms and/or their metabolites (Ross *et al.*, 2002). The potential of biopreservation by lactic acid bacteria, including *Lb. sakei*, has been reviewed for seafood (Ghanbari *et al.*, 2013), vegetable foods (Settani & Corsetti, 2008) and meat (Castellano *et al.*, 2008). The species *Lb. sakei* has been divided in two subspecies, *Lb. sakei* subsp. *sakei* and *Lb. sakei* subsp. *carnosus*, however a recent study has suggested a profound revision of the subspecies definition based on multilocus sequence typing (MLST) data (Chaillou *et al.*, 2013). *Lb. sakei* has the qualified presumption of safety (QPS) status. This involves a safety assessment of biological agents intentionally added to food and feed based on four pillars: establishing identity, body of knowledge, possible pathogenicity and end use. A generic qualification for all bacterial taxonomic units on the QPS recommended list is the absence of any acquired antimicrobial resistance genes to clinically relevant antibiotics (EFSA, 2012). However, the presence of antibiotic resistance genes located on mobile genetic elements has been demonstrated for some *Lb. sakei* strains (Gevers *et al.*, 2003a; Ammor *et al.*, 2008). Concerning lactic acid bacteria, there is increasing evidence that they might play an important role as reservoir of potentially transferable antibiotic resistance genes (reviewed by Devirgiliis *et al.*, 2013). An increasing number of foodborne *Lactobacillus* species carrying one or more antibiotic resistance genes has been reported. The association of these resistance genes with mobile elements as well as their possible horizontal transfer were however not always investigated, but if so tetracycline and erythromycin resistance were implicated (Devirgiliis *et al.*, 2013).

3.3. Biofilms

In the food industry, biofilms represent a substantial problem. A biofilm can be defined as "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription" (Donlan & Costerton, 2002). Biofilms in the food industry can lead to high costs due to their possible contribution to food spoilage, equipment failure

and dissemination of pathogens. Bacterial biofilm formation can be considered as the result of an interplay between features of the bacterial cells, the substratum and the surrounding environment. During biofilm formation five stages can be distinguished: I) initial attachment, II) irreversible attachment, III) early development of biofilm architecture, IV) maturation, and V) dispersion (Figure 1.5). In the following section, a short overview of the basic principles of the different stages is presented (Cloete *et al.*, 2009). The transition from planktonic growth to biofilm formation is induced by changes of environmental conditions and is associated with alterations in gene regulation. Before biofilm formation takes place, conditioning of the surface often occurs, which implies the formation of a film of organic molecules in fluid surroundings causing changes in the properties of the substratum. Initial attachment can only take place once the organism is in close proximity of the substratum. This can be directed passively by gravity, diffusion and fluid dynamics or actively by bacterial cell surface properties (Chmielewski & Frank, 2003). Weak forces, such as van der Waals and electrostatic forces and hydrophobic interactions are involved in this initial and reversible stage of biofilm formation. The second stage, irreversible attachment, also called the anchoring or locking stage is assisted by bacterial motility structures, such as flagella and pili, bacterial surface proteins and the production of EPS. After this irreversible attachment, development and maturation of the biofilm is ensured by genotypic and phenotypic changes. Microcolonies are formed when the bacteria start to multiply within the EPS. Further development of microcolonies gives rise to macrocolonies. These contain a larger number of cells, are divided by fluid-filled channels and demonstrate a higher metabolic and physiological heterogeneity, but are still enclosed in the EPS. The number of cells can increase in the colonies by translocation of cells on the surface, by the direct attachment of planktonic cells or by cell division. Biofilm maturation begins after irreversible attachment is established and implies an increase in overall density and complexity of the biofilm structure. Detachment and dispersal are necessary for survival and colonization of new niches. Erosion and sloughing are two spontaneous detachment processes. Erosion is the continual detachment of small portions or single cells from the biofilm, whereas sloughing implies the rapid loss of large portions. Other detachment processes are collisions between biofilm carriers (abrasion), human intervention or grazing.
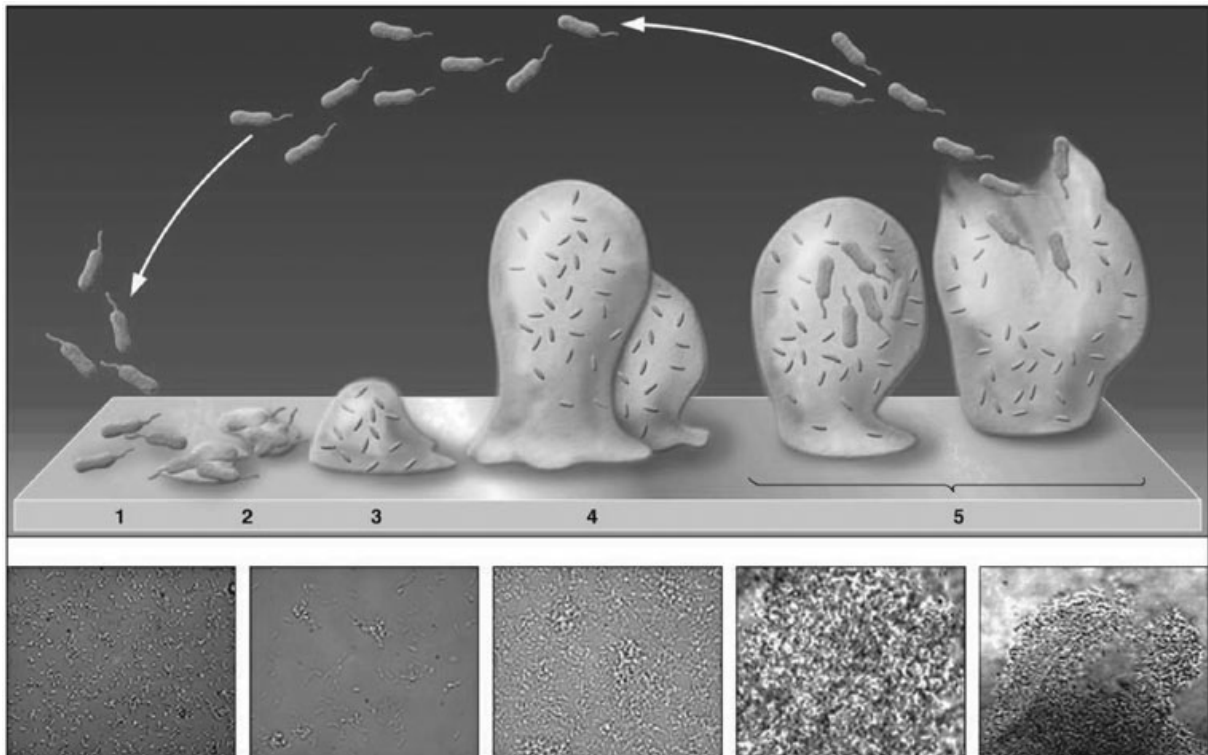
Figure 1.5. *Stages of bacterial biofilm formation. 1) initial attachment, 2) irreversible attachment, 3) early development of biofilm architecture, 4) maturation, and 5) dispersion. (Source: Marchand* et al.*, 2012)*

Biofilms demonstrate high antibiotic tolerance and resistance, which is caused by an interplay of different mechanisms, such as restricted penetration, reduced growth rate, metabolic heterogeneity within the biofilm, general stress response, the presence of persister cells (*i.e.* dormant variants of regular cells that exhibit multidrug tolerance (Lewis, 2010)), a specific biofilm phenotype, quorum sensing, efflux pumps (Mah & O'Toole, 2001; Stewart, 2002; Drenkard, 2003; de la Fuente-Núñez *et al.*, 2013). There is a fundamental difference between antibiotic tolerance and antibiotic resistance. Antibiotic tolerance is a property of dormant cells that survive killing by bactericidal antibiotics in the absence of drug resistance mechanisms, while in antibiotic resistance the interaction of an antibiotic with a target is prevented by a variety of resistance mechanisms (Lewis, 2010). Recent studies have demonstrated that the transfer of antibiotic resistance is higher under biofilm conditions than under planktonic conditions (Hennequin *et al.*, 2012; Savage *et al.*, 2013). However, biofilms are not only hotspots for horizontal gene transfer, but there is also an interconnection between biofilm formation and stabilization and horizontal gene transfer (reviewed by Molin & Tolker-Nielsen, 2003 and by Madsen *et al.*, 2012). Several studies have demonstrated that the presence of conjugative plasmids in Gram-negative bacteria stimulates biofilm formation

(Ghigo, 2001; Dudley *et al.*, 2006; Reisner *et al.*, 2006; Burmølle *et al.*, 2008). Pili or fimbriae encoded on the plasmid seem to be responsible, like for example the F conjugative pili as demonstrated by Ghigo (2001), the type IV pilus as demonstrated by Dudley *et al.* (2006) or the type 3 fimbriae as demonstrated by Burmølle *et al.*, 2008. In Gram-positive bacteria, which apply other conjugative mechanisms than Gram-negative bacteria, the stimulating effect of conjugation on biofilm formation has also been demonstrated (Luo *et al.*, 2005). The presence of a conjugative plasmid however, may also have a host-specific negative effect on biofilm formation on solid, abiotic surfaces. The study of Røder *et al.* (2013) indicated that the presence of conjugative plasmids in some species may facilitate tight cell–cell attachment, favoring the formation of cell aggregates (flocs) over biofilm formation.

3.4. *Processing*

One of the techniques that is being applied to extend the shelf life of food products is modified atmosphere packaging (MAP). MAP aims to reduce or inhibit bacterial growth. The application of MAP has been reviewed for a wide variety of food products, such as meat, fish and fishery products, fruits and vegetables, dairy products, grains and mushrooms (*e.g.* Jayas & Jeyamkondan, 2002; Sivertsvik *et al.*, 2002; Palacios *et al.*, 2011; Arvanitoyannis & Stratakos, 2012; Singh *et al.*, 2012; Caleb *et al.*, 2013). Some of these reviews also took into consideration the safety concern related to MAP products as this technique could possibly enable the growth of psychrotrophic pathogens. The gasses mostly applied in MAP are oxygen (O_2), nitrogen (N_2) and carbon dioxide (CO_2). Oxygen is mainly being used in MAP of fresh fruits and vegetables to allow these products to respire and in MAP of meat products to maintain the red color (Church & Parson, 1995). Oxygen at superatmospheric partial pressure (> 70 kPa O_2) may inhibit, have no effect, or even stimulate growth of different microorganisms from the same genus (Artés & Allende, 2005). Nitrogen is used as a filler gas. Although it has no antimicrobial effect itself, it prevents the growth of aerobic organisms by replacing O_2. Carbon dioxide has a strong antimicrobial effect causing an extension of the lag phase and a reduction in growth rate during the logarithmic phase (Farber, 1991). Several underlying mechanisms have been suggested: change in cell-membrane function, direct enzyme inhibition or reduced enzyme reaction rates, intracellular pH changes caused by penetration of bacterial membranes, alterations of physicochemical properties of cell proteins (Farber, 1991). In general, Gram-negative bacteria are more sensitive to CO_2 than Gram-positive bacteria (Church, 1994). As the solubility of CO_2 decreases dramatically with

increasing temperatures, temperature plays an important role in the effect of CO_2 (Farber, 1991).

Low temperature itself can also be applied as a preservation technique. Reducing temperature slows the rates of chemical reactions and the growth of microorganisms (Farkas, 2007). Bacteria are classified based on their optimal growth temperature, namely the psychrophiles, the mesophiles and the thermophiles. A special group are the psychrotrophs which are able to grow at temperatures between 0 and 7 °C, although they are in fact mesophiles (Jay *et al.*, 2005). An important psychrotropic foodborne pathogen is *L. monocytogenes*. In general, it grows very slow at temperatures below 4 °C. When the temperature however rises above 4 °C, the growth rate increases and the lag phase decreases considerably. The risk that comes along with storage at slight abuse temperatures is therefore increased as *L. monocytogenes* can grow to numbers which pose a possible threat to human health.

Low temperature can also have an influence on antibiotic resistance transfer, as has been mentioned in section 2.3.

4. Objectives

The spread of antibiotic resistance results from an interplay of factors between humans, animals, food and environment. In case of antibiotic resistance transfer to humans through food, the food production chain does not only play a passive role but it also affects survival and growth of antibiotic resistant bacteria and transfer of antibiotic resistance genes.

This PhD research aims at providing more insight into the acquisition and dissemination of antibiotic resistance during food production and preservation as this has not yet been studied extensively. This was done by applying several quantification techniques (plating and flow cytometry), using both a Gram-negative as a Gram-positive model system, with bacteria having a specific role in the food industry and by considering food production and food preservation aspects (Figure 1.6).

The aim of **Chapter 2** was to study the transfer of a multiresistance plasmid, which was originally isolated from a wastewater treatment plant, to the foodborne pathogens, *Salmonella* spp. and *E. coli* O157:H7. Two quantification methods, plating and flow cytometry, were applied. Antibiotic resistance profiles of recipients and transconjugants were determined.

Integrons represent an interesting mechanism by which bacteria can capture antibiotic resistance genes. In **Chapter 3**, the presence of integrons in a Belgian collection of Shiga-toxin producing *E. coli*, the most significant group of emerging foodborne pathogens, was explored and further characterized. The antibiotic resistance of integron-positive and -negative strains was compared.

Biofilms are a significant problem in the food industry. It is therefore important to analyze to which extent and at which frequencies plasmid transfer may occur in these structures. This was the objective of **Chapter 4**, using a reactor in which three biofilm models, representative for biofilms in the food industry, were integrated. *P. putida*, a model for spoilage organisms, was used as donor and *E. coli*, a model for foodborne pathogens, as recipient.

The food industry increasingly uses minimal processing techniques to provide the consumer high quality food, which also possesses a sufficiently long shelf life. Techniques often used to achieve this are low temperature and modified atmosphere packaging. How these techniques influence plasmid transfer was the subject of **Chapter 5**. A Gram-positive model was used

with the donor *Lb. sakei* subsp. *sakei*, as model for spoilage organisms and the well-known foodborne pathogen *L. monocytogenes* as recipient. Cooked ham was used for the validation of plasmid transfer under MAP. In a last step, low inoculum densities were applied to approach a more realistic situation.

In **Chapter 6** the obtained results are discussed in light of the farm to fork concept, starting from the primary production up to the consumer, followed by a discussion on the methodology. Finally, some conclusions are drawn.
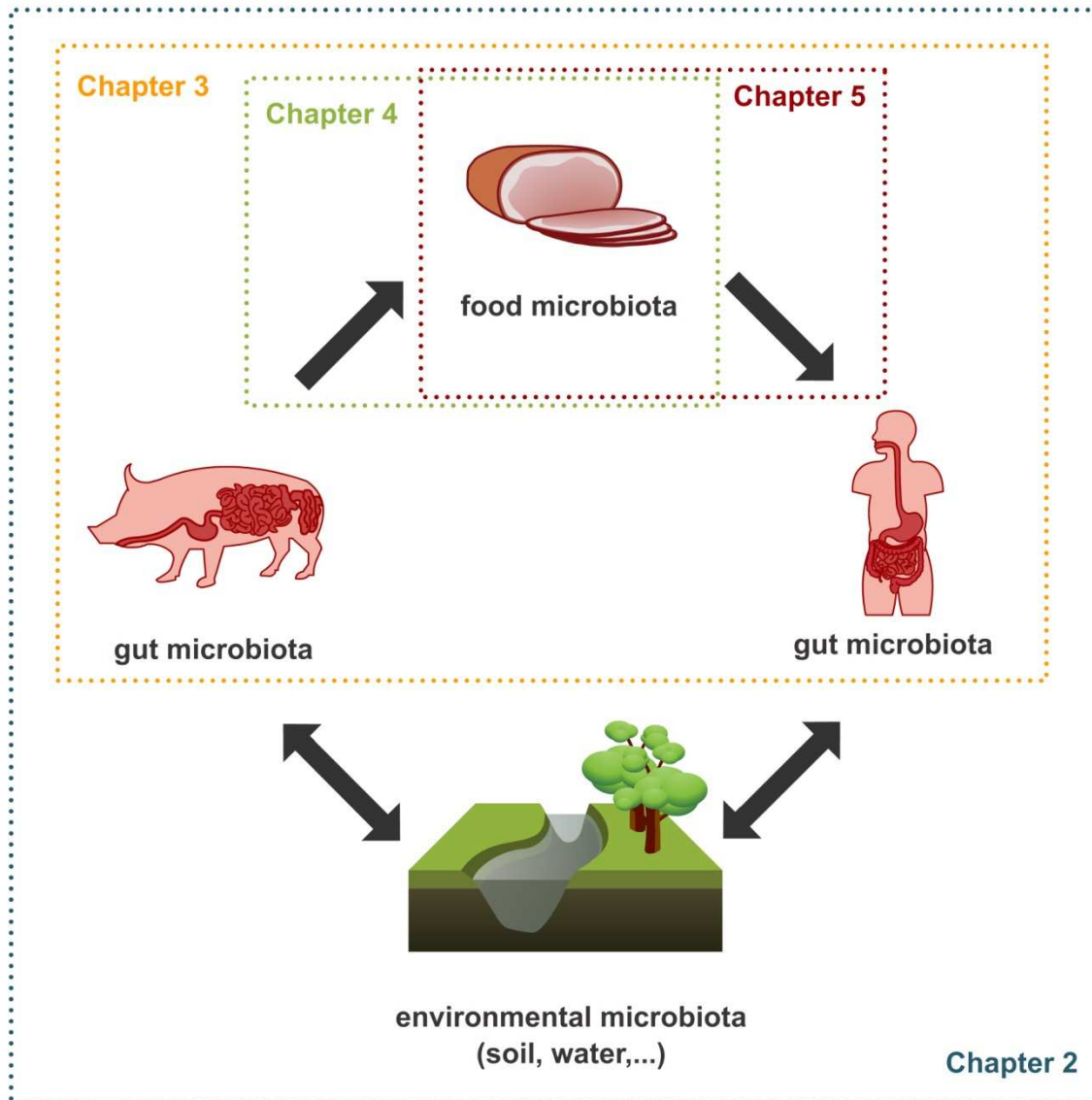
Figure 1.6. *Schematic overview of the different research chapters. The spread of antibiotic resistance results from an interplay of different factors. In this doctoral work, the interplay between environment, primary production, food processing and consumer was considered. In* ***Chapter 2****, the transfer of an environmental plasmid to foodborne pathogens, isolated from humans, food or animals was analyzed (Model organisms:* P. putida, Salmonella *spp. and* E. coli *O157:H7). In* ***Chapter 3****, a collection of Belgian Shiga toxin producing E. coli, isolated from humans, food or animals was screened for the presence of integrons. In* ***Chapter 4****, plasmid transfer in biofilm models representative for the food industry was examined (Model organisms:* P. putida *and* E. coli*). In* ***Chapter 5****, the influence of preservation techniques on plasmid transfer was explored (Model organisms:* Lb. sakei *subsp.* sakei *and* L. monocytogenes*).*

The topic of this chapter is the interplay between environment and foodborne pathogens. The transfer of a multiresistance plasmid, originally isolated from a wastewater treatment plant, to foodborne pathogens was investigated by plating and flow cytometry. Subsequently, the expression of the acquired resistance genes in the transconjugants was inspected.

Chapter 2

Strain-specific transfer of antibiotic resistance from an environmental plasmid to foodborne pathogens

Abstract

Pathogens resistant to multiple antibiotics are rapidly emerging, entailing important consequences for human health. This study investigated if the broad-host-range multiresistance plasmid pB10, isolated from a wastewater treatment plant, harbouring amoxicillin, streptomycin, sulfonamide and tetracycline resistance genes, was transferable to the foodborne pathogens *Salmonella* spp. or *E. coli* O157:H7 and how this transfer alters the phenotype of the recipients. The transfer ratio was determined by both plating and flow cytometry. Antibiotic resistance profiles were determined for both recipients and transconjugants using the disk diffusion method. For 14 of the 15 recipient strains, transconjugants were detected. Based on plating, transfer ratios were between 6.8×10^{-9} and 3.0×10^{-2} while using flow cytometry, transfer ratios were between $<1.0 \times 10^{-5}$ and 1.9×10^{-2}. With a few exceptions, the transconjugants showed phenotypically increased resistance, indicating that most of the transferred resistance genes were expressed. In summary, we showed that an environmental plasmid can be transferred into foodborne pathogenic bacteria at high transfer ratios. However, the transfer ratio seemed to be recipient strain dependent. Moreover, the newly acquired resistance genes could turn antibiotic susceptible strains into resistant ones, paving the way to compromise human health.

1. Introduction

The extensive use of antibiotics in human and veterinary medicine and its prophylactic and growth promoting use in agriculture and aquaculture have lead to a huge rise of antibiotic resistant bacteria (Hamer & Gill, 2002; Cabello, 2006; Walsh & Fanning, 2008) and an increase of antibiotic resistance genes in the horizontal gene pool.

Antibiotic resistance in bacteria can be intrinsic or acquired. In the case of intrinsic resistance, bacterial strains are inherently resistant to a certain compound and the resistance cannot be transferred horizontally (Fajardo *et al.*, 2008). Acquired resistance occurs by mutation and/or horizontal gene transfer events. The main mechanisms of horizontal gene transfer are conjugation (mobile genetic elements are being transferred from a donor to a recipient cell), transformation (uptake of naked DNA) and transduction (bacteriophages as transporters of genetic information). Conjugation is considered as the principal mode for antibiotic resistance transfer since many antibiotic resistance genes are situated on mobile elements such as plasmids and conjugative transposons. Conjugation of broad-host-range plasmids enables DNA to be transferred over genus and species borders, whereas transformation and transduction are usually more limited to the same species (Mathur & Singh, 2005). When considering a medical point of view, the transfer of antibiotic resistance determinants from environmental bacteria to pathogens is of utmost importance, and it is clear that environmental bacteria should not be seen as devoid of antibiotic resistance determinants because of the physical distance between these bacteria and clinical settings (Moore *et al.*, 2010). A recent study suggests that infected patients might enhance the spread of plasmid-encoded fitness, virulence and antibiotic resistance determinants as inflammation elicits concomitant *Salmonella* and *Escherichia coli* blooms, which can strongly raise donor and recipient densities in the gut, thereby boosting horizontal gene transfer (Stecher *et al.*, 2012).

The aim of this study was to investigate if an environmental multiresistance plasmid can be transferred to two model Gram-negative foodborne pathogens, that are, *Salmonella* spp. and *E. coli* O157:H7. It is generally agreed that Gram-negative bacteria pose the greatest risk to public health as the increase in resistance of Gram-negative bacteria is faster than in Gram-positive bacteria and as there are fewer new and developmental antibiotics active against Gram-negative bacteria (Kumarasamy *et al.*, 2010).

To determine the transfer ratio, the transconjugants were analyzed by both plating and flow cytometry (*gfp* as the reporter gene). The application of flow cytometry for the detection and

quantification of plasmid transfer was first described and evaluated by Sørensen *et al.* (2003). Since then, this technique has been applied for the quantification of transconjugants in other studies (Boon *et al.*, 2006; Musovic *et al.*, 2006; Shintani *et al.*, 2014). The extent to which the phenotype of the transconjugants was influenced, was analyzed by determining the antibiotic resistance profiles against five antibiotics for the recipients and the transconjugants.

2. **Material and Methods**

2.1. Bacterial strains, plasmid and growth conditions

The plasmid donor strain was *Pseudomonas putida* strain SM1443, a KT2442 (SM1315) strain with the mini-Tn*5*-*lacI*^{q} cassette inserted into the chromosome (Christensen *et al.*, 1998). The *lacI*^{q} repressor cassette prevented the expression of the *gfp* gene in the donor (Figure 2.1).

The plasmid used in this study was the broad-host-range plasmid pB10. This plasmid, belonging to the IncP-1β subgroup, was isolated from a wastewater treatment plant and contains resistance to the antibiotic agents amoxicillin, streptomycin, sulfonamides and tetracycline and to inorganic mercury ions (Dröge *et al.*, 2000). To mark the plasmid with a *gfp* gene and a *npt* gene (kanamycin resistance gene, Km) (Figure 2.1), insertion of the mini-Tn*5*-Km-P$_{A1-04/03}$::*gfp* cassette was performed in two steps. First, a triparental mating was performed in which the helper plasmid RK600 (Kessler *et al.*, 1992), present in *E. coli* HB101, mobilized the delivery plasmid pJBA120, containing the mini-Tn*5* cassette, from the donor *E. coli* MV1190(λ-*pir*) (Andersen *et al.*, 1998), into the rifampicin resistant recipient *P. putida* UWC1 harbouring pB10. *P. putida* UWC1 derivatives with the mini-Tn*5*-Km-P$_{A1-04/03}$::*gfp* cassette inserted either in the chromosome or in pB10 were obtained by selection in Luria Bertani (LB) broth (10 g tryptone, 5 g yeast extract and 5 g NaCl per litre) with 10 µg tetracycline mL^{-1}, 50 µg kanamycin mL^{-1} and 100 µg rifampicin mL^{-1}. In the second step, *gfp*-marked plasmids were obtained by mating the *P. putida* UWC1 derivatives with *Ralstonia eutropha* JMP228n (De Gelder *et al.*, 2005). Selection on LB agar plates with 10 µg tetracycline mL^{-1}, 50 µg kanamycin mL^{-1} and 100 µg nalidixic acid mL^{-1} resulted in JMP228n clones carrying pB10 containing a randomly inserted mini-Tn*5*-Km-P$_{A1-04/03}$::*gfp* cassette. Subsequently, one clone, designated JMP228n (pB10::*gfp*), was mated with *E. coli* MG1655 to obtain *E. coli* MG1655 (pB10::*gfp*) after selection on LB agar plates with 10 µg tetracycline mL^{-1} and 50 µg kanamycin mL^{-1} at

43 °C. Ultimately, this strain was mated with *P. putida* SM1443 to obtain the donor strain for the experiments, *P. putida* SM1443 (pB10::*gfp*), after selection on LB agar plates with 10 µg tetracycline mL^{-1}, 100 µg rifampicin mL^{-1} and 50 µg kanamycin mL^{-1} at 28 °C.
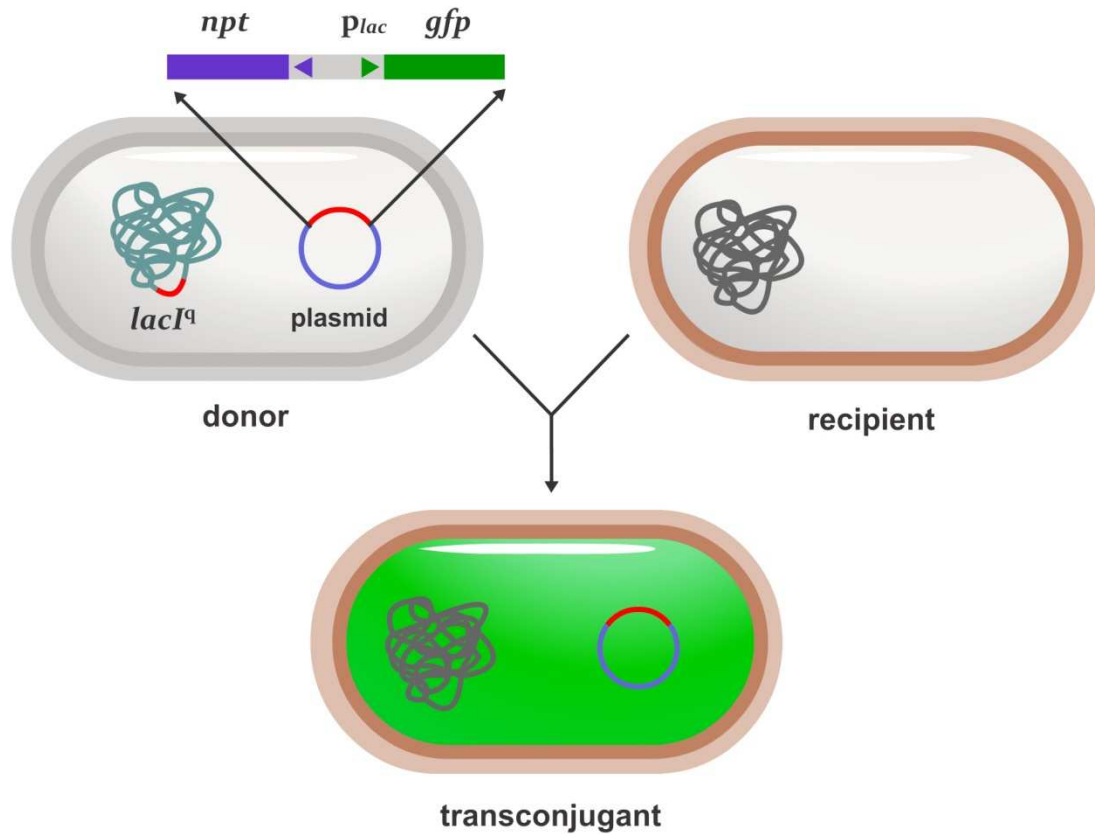
Figure 2.1. *Principle of the* gfp *reportersystem. The donor strain contains chromosomally a* lacI^q *repressor preventing the expression of the* gfp *gene located on the plasmid. After plasmid transfer the* gfp *gene is expressed in the transconjugant cell, which consequently can be detected by flow cytometry.*

The recipient strains were 10 *Salmonella* spp. and five *E. coli* O157:H7 strains (Table 2.1). The tested S*almonella* serovars belong to the most frequently occurring *Salmonella* serotypes in human salmonellosis in Europe, with *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Salmonella enterica* subsp. *enterica* serovar Typhimurium being the most frequent (EFSA/ECDC, 2010). None of the five *E. coli* O157:H7 strains carried *stx*1 and *stx*2 genes. For one strain (LFMFP 476), no additional information on the presence of other virulence genes was available, but the four other strains all carried the *eae* and *ehx* genes.

The recipient strains were first tested on their inability to grow on kanamycin (50 µg mL^{-1}) containing plates as this antibiotic was used as selective marker to detect transconjugants harbouring pB10::*gfp*.

Donor and recipient strains were all grown in LB broth. For all solid media, 1.5% agar was added. *P. putida* was incubated at 28 °C, *Salmonella* spp. and *E. coli* at 37 °C. To maintain the plasmid in the donor and the transconjugants 50 µg kanamycin mL^{-1} was added to the medium.

Table 2.1. *Overview of the recipient strains (ILVO laboratory collection and LFMFP laboratory collection).*

Strain	Species	Serovar / Serotype	Origin
MB 1139	*Salmonella enterica*	Enteritidis	Poultry
MB 1410	*Salmonella enterica*	Enteritidis	Egg
MB 1561	*Salmonella enterica*	Enteritidis	Poultry (transport)
MB 2264	*Salmonella enterica*	Typhimurium	Human
MB 2265	*Salmonella enterica*	Typhimurium	Human
MB 2272	*Salmonella enterica*	Typhimurium	Human
MB 2292	*Salmonella enterica*	Typhimurium	Human
MB 1641	*Salmonella enterica*	Hadar	Poultry (cecal drop)
KS 1-1	*Salmonella enterica*	Infantis	Poultry (house)
KS 87	*Salmonella enterica*	Virchow	Poultry (house)
MB 3885	*Escherichia coli*	O157:H7	Beef (carpaccio)
MB 3890	*Escherichia coli*	O157:H7	Human
MB 4021	*Escherichia coli*	O157:H7	Bovine (carcass)
MB 4260	*Escherichia coli*	O157:H7	Non-human
LFMFP 476	*Escherichia coli*	O157:H7	Bovine (faeces)

2.2. Filter mating

Mating experiments were conducted in triplicate (biological replicates) on 0.22 µm polycarbonate filters (25 mm diameter) (Whatman, UK). The donor and recipient cultures were grown overnight and washed twice with sterile saline (0.85% NaCl) to remove antibiotics. The OD$_{610nm}$ was adjusted to 0.25-0.35 (approximately 10^8 cells mL^{-1}) for both donor and recipient strains. Seventy-five µL of both donor and recipient was diluted in 2 mL of sterile saline and distributed evenly over the filter using a Swinnex device (Millipore, USA). The filters were transferred to LB agar plates and incubated overnight at 28 °C. Afterwards, the filters were submerged in 5 mL sterile saline and vortexed twice for 1 min. The suspended bacteria were analyzed by plate counting (n = 1) and by flow cytometry

(n = 3). For the plate counting, LB plates, which contained kanamycin, were incubated at 42 °C. The presence of the antibiotic counter selected for the recipient strain, while the high temperature counter selected for the donor strain. The transfer ratio was determined as the number of transconjugant CFU per total cell count (donor, recipient and transconjugant cells), as determined by flow cytometry.

2.3. Flow cytometry analysis

Diluted bacteria were detected and quantified with a Cyan ADP Flow Cytometer (Dako, Denmark), using the 488 nm laser. The dilution factor ranged from 1000 to 2500. Dilutions were made with filter sterilized Evian water. Each sample consisted of 980 µL of the diluted sample, 10 µL Na_2EDTA (500 mM, pH8) and 10 µL Dako Cytocount beads. These beads were used to determine the cell concentration. Green fluorescence emission was collected with a photomultiplier tube using a 530/40 emission filter, for PE 585 fluorescence a 575/25 emission filter was used and side scatter light (SSC) was collected using a 488/10 emission filter. The sheath fluid consisted of Milli-Q water. The threshold trigger was set to SSC. The analysis of a sample was done by collecting data for 100 000 events in threefold. Summit v4.3 software was used to process the results. Pure cultures of donor, recipient and transconjugant were analyzed by flow cytometry to set the gate that distinguish between the transconjugant population and the donor and recipient population on a Green Log versus PE 585 Log plot. When the transconjugants of a specific filter mating sample could not clearly be visually detected on the plot, their number was considered to be below the detection limit ($<1 \times 10^{-5}$ transconjugants per total cells). The transfer ratio was determined as the number of transconjugant cells per total cell count.

2.4. Antibiotic susceptibility screening

The antibiotic susceptibility of the recipients and transconjugants was determined by using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for five antibiotics (amoxicillin, kanamycin, streptomycin, sulfonamides and tetracycline) (CLSI, 2009). The visual turbidity of the bacterial isolates was adjusted to a 0.5 McFarland standard in sterile saline. The suspension was plated on a Mueller-Hinton agar plate (Oxoid, UK) and antibiotic disks (Oxoid) were applied on the plate. Inhibition zone diameters were measured after incubating the plates during 16-18 h at 37 °C. Classification as "susceptible", "intermediate resistant" or "resistant" was based on the inhibition zone

diameters according to CLSI guidelines. *E. coli* ATCC 25922 was used as quality control strain to monitor the performance of the susceptibility testing.

2.5. *Molecular confirmation of plasmid transfer*

Transfer of the plasmid pB10::*gfp* was confirmed by PCR. DNA from the recipient and transconjugant strains was obtained by an alkaline lysis method. For each strain, a few bacterial colonies were suspended in 1 mL Ringer solution. After centrifuging the sample for two minutes at 14000 *g*, 100 µL sterile water was added to the pellet. The samples were incubated for 15 minutes at 90 °C and subsequently centrifuged for one minute at 14000 *g*. Fifty µL of the supernatant was kept at -20 °C.

The PCR reaction was performed with the primers trfA_fw and trfA_rev to amplify a 281 bp fragment of the replication initiation gene *trfA*, encoded by the plasmid, as previously described (Bahl *et al.*, 2009). These primers are specific for plasmids belonging to the IncP-1α, β, ε subgroups. The PCR amplification products were detected by electrophoresis on a 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer and visualized by ethidium bromide staining.

3. Results

3.1. *Characterization of the recipient strains*

Before starting the conjugation experiments, the antibiotic susceptibility profiles of the recipient strains and presence of IncP-1α, β, ε plasmids were determined (Table 2.2). The three *S.* Enteritidis strains were susceptible to the tested antibiotics, except one (MB 1139), which displayed an intermediate resistance to kanamycin. There was much more variation in the antibiotic susceptibility profiles of the *S. enterica* serovar Typhimurium strains. The *S. enterica* serovar Typhimurium strain MB 2264 was resistant to the four antibiotics which are indigenous to the plasmid but susceptible to kanamycin, while *S. enterica* serovar Typhimurium strain MB 2265 was susceptible to all the antibiotics. The two other *S. enterica* serovar Typhimurium strains (MB 2272 and MB 2292) showed resistance to, respectively, one (amoxicillin) and two antibiotics (amoxicillin and sulfonamides). The *Salmonella enterica* subsp. *enterica* serovar Hadar strain MB 1641 was susceptible to kanamycin and sulfonamides. The strains of *Salmonella enterica* subsp. *enterica* serovar Infantis KS 1-1 and *Salmonella enterica* subsp. *enterica* serovar Virchow KS 87 were susceptible to all five

antibiotics. All the recipient *E. coli* strains were susceptible to the antibiotics tested, except strain MB 3890 which was intermediate resistant to streptomycin.

The absence of IncP-1α, β, ε plasmids in the recipient strains was confirmed by PCR as in none of the 15 recipient strains a PCR fragment of 281 bp, specific for IncP-1α, β, ε plasmids, was detected (data not shown).

3.2. *Plasmid transfer analyzed by plating*

Suspensions, obtained after filter mating, were plated on LB plates supplemented with kanamycin and incubated at 42 °C. Transconjugants were obtained for 13 of the 15 tested strains (Figure 2.2). The strains that did not yield transconjugants, were *S. enterica* serovar Enteritidis MB 1139 and *S. enterica* serovar Hadar MB 1641. Repetition of the conjugation experiments confirmed these results (data not shown). The other *Salmonella* spp. strains resulted in transfer ratios ranging from 3.7×10^{-7} to 3.0×10^{-2} transconjugants per total cell count. The highest transfer ratios were found for the two remaining *S. enterica* serovar Enteritidis strains (MB 1561: 3.0×10^{-2}; MB 1410: 9.1×10^{-4}), followed by *S. enterica* serovar Virchow KS 87 (7.2×10^{-4}) and *S. enterica* serovar Infantis KS 1-1 (9.2×10^{-5}), while the lowest transfer ratios were observed for the *S. enterica* serovar Typhimurium strains, with transfer ratios in the order of 10^{-7}. For MB 2265 a transfer ratio of 1.9×10^{-5} was observed, which was the fifth highest transfer ratio found for the *Salmonella* spp. strains tested. One of the *E. coli* strains (MB 3890) had a similar transfer ratio (2.2×10^{-5}) as some *Salmonella* spp. strains, while the other four *E. coli* strains had much lower transfer ratios ($10^{-8} – 10^{-9}$).

Table 2.2. *Antibiotic susceptibility screening by disk diffusion of the recipients (R) and the transconjugants (T). Values represent inhibition zone diameters (mm)*

Strain	Kanamycin		Amoxicillin		Streptomycin		Sulfonamides		Tetracycline	
	R	T	R	T	R	T	R	T	R	T
S. enterica serovar Enteritidis (MB 1139)	*17*	*	28	*	15	*	20	*	23	*
S. enterica serovar Enteritidis (MB 1410)	20	<7	20	7	17	*13*	20	<7	21	<7
S. enterica serovar Enteritidis (MB 1561)	21	<7	27	7	18	*13*	22	<7	22	<7
S. enterica serovar Typhimurium (MB 2264)	19	<7	<7	<7	**8**	**8**	<7	<7	**9**	<7
S. enterica serovar Typhimurium (MB 2265)	20	<7	26	7	15	*14*	20	<7	21	<7
S. enterica serovar Typhimurium (MB 2272)	20	<7	<7	<7	15	*12*	25	<7	21	<7
S. enterica serovar Typhimurium (MB 2292)	19	<7	<7	<7	15	*13*	<7	<7	20	<7
S. enterica serovar Hadar (MB 1641)	18	*	<7	*	**9**	*	21	*	**< 7**	*
S. enterica serovar Infantis (KS 1-1)	19	<7	25	7	15	*12*	19	<7	19	<7
S. enterica serovar Virchow (KS 87)	19	<7	26	7	15	*12*	21	<7	20	<7
E. coli O157:H7 (MB 3885)	21	<7	22	7	16	*13*	24	<7	22	<7
E. coli O157:H7 (MB 3890)	18	<7	21	7	*14*	*14*	24	<7	20	<7
E. coli O157:H7 (MB 4021)	21	<7	21	7	18	15	24	<7	21	<7
E. coli O157:H7 (MB 4260)	19	<7	20	7	15	*13*	24	<7	20	<7
E. coli O157:H7 (LFMFP 476)	19	<7	22	**11**	15	*14*	24	<7	21	<7

Bold: considered as resistant according to CLSI guidelines.
Italic: considered as intermediate resistant according to CLSI guidelines.
*: no transconjugants obtained.
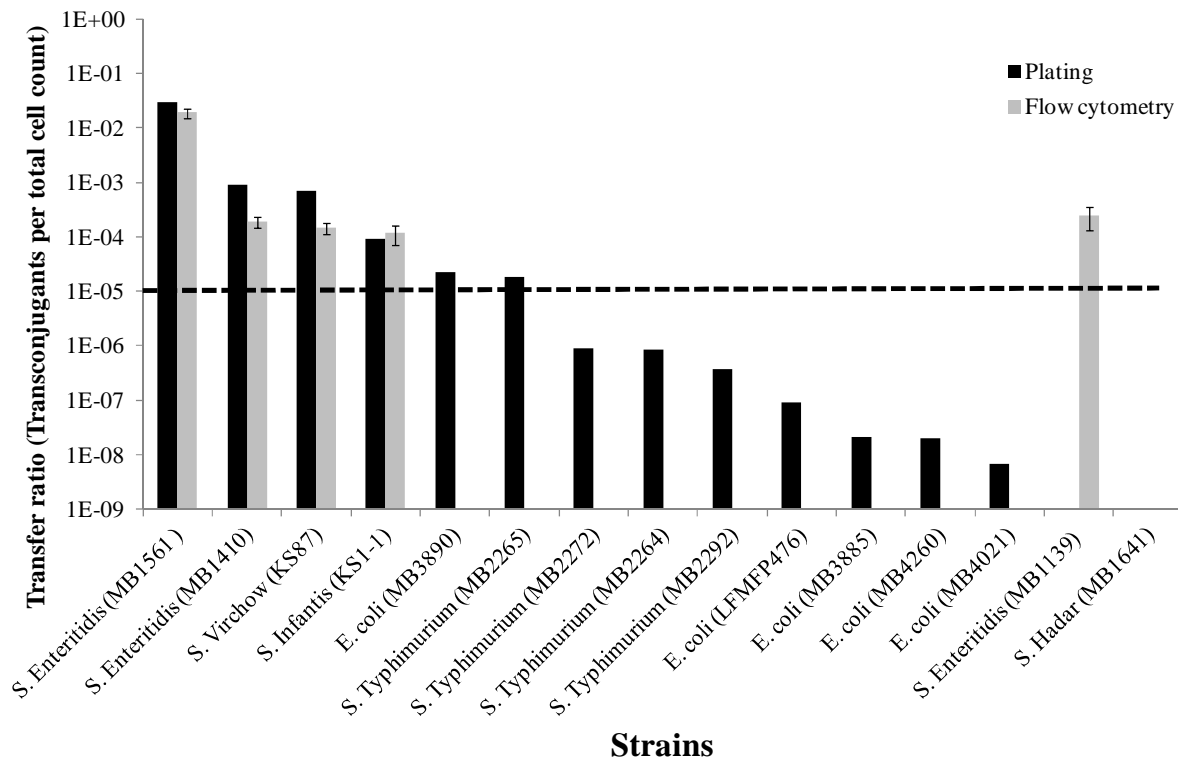
Figure 2.2. *Transfer ratio, expressed as number of transconjugants per total cell count, determined by plating (n = 1) (black bars) and by flow cytometry (n = 3) (grey bars) for the 15 recipient strains. The dashed line represents the detection limit of flow cytometry.*

3.3. Plasmid transfer analyzed by flow cytometry

The conjugation efficiency was also assessed by flow cytometry, because this method allowed a rapid and culture-independent screening of the individual transconjugant and parental cells (Figure 2.3). Using the same mating mixtures as described above, transconjugants could be detected for only five of the 15 tested strains, due to the rather poor detection limit (Figure 2.2). These strains were all *Salmonella* spp., more specifically *S. enterica* serovar Enteritidis (MB 1561: 1.9×10^{-2}; MB 1139: 2.5×10^{-4}, MB 1410: 1.9×10^{-4}), *S. enterica* serovar Virchow (1.5×10^{-4}) and *S. enterica* serovar Infantis (1.2×10^{-4}). No transconjugants could be obtained by plating for *S. enterica* serovar Enteritidis MB 1139, while the four other strains showed the highest transfer ratio determined by plating. For the 10 other strains the transfer ratio was below the detection limit ($<1 \times 10^{-5}$ transconjugants per total cell count). This is consistent with the low transfer ratios obtained by plating ($10^{-5} – 10^{-9}$).
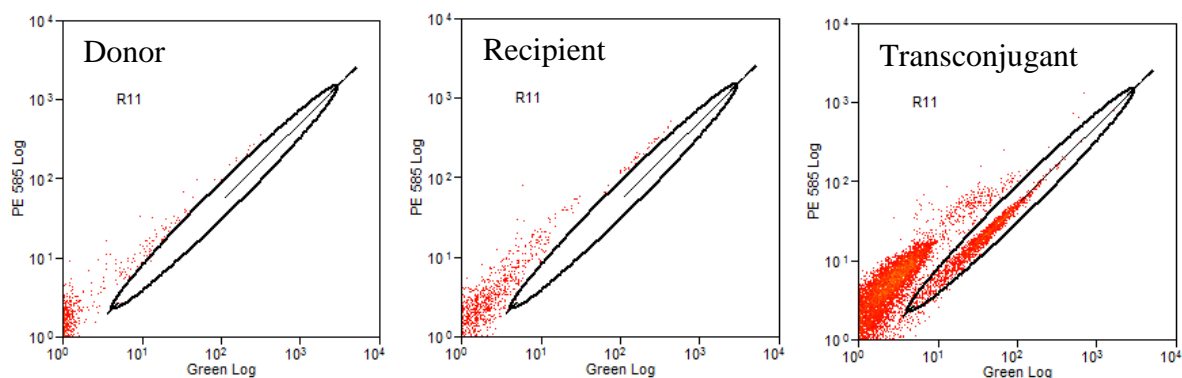
Figure 2.3. *Detection of transconjugants by flow cytometry. By analyzing pure cultures of donor, recipient and transconjugant a gate was set on a Green Log versus PE 585 Log plot to distinguish transconjugants.*

3.4. Characterization of the transconjugants

To confirm that plasmid transfer had occurred and to analyze which effect this transfer had on the phenotype, the presence of the plasmid in the transconjugants and the antibiotic resistance profiles of the transconjugants were examined. Transconjugants were obtained for 13 of the 15 tested strains by plating (Table 2.2). As expected, the transconjugants were all resistant to kanamycin (inhibition zone diameter <7 mm). The inhibition zone diameter of sulfonamides and tetracycline was less than 7 mm for all the transconjugants, meaning that they were all completely resistant to these compounds. For amoxicillin the inhibition zone diameter was 7 mm or less, except for *E. coli* LFMFP 476 for which the inhibition zone diameter was 11 mm. This value is still considered as resistant according to CLSI guidelines. The decrease in inhibition zone diameter was less pronounced for streptomycin. According to the CLSI guidelines 11 of the transconjugant strains are considered to be intermediate resistant to streptomycin, one *E. coli* strain (MB 4021) remained susceptible. *S. enterica* serovar Typhimurium MB 2264 was already resistant to amoxicillin, streptomycin, sulfonamides and tetracycline before conjugation. Phenotypically, this strain gained only the resistance to kanamycin upon conjugation.

The presence of the pB10 plasmid in the transconjugants was confirmed by PCR. While none of the recipient strains contained the fragment (see above), the transconjugant strains all showed a clear band of the expected size after gel electrophoresis (data not shown).

4. Discussion

This study demonstrated that the broad-host-range plasmid pB10, carrying multiple resistance genes, could be transferred to foodborne pathogens under laboratory conditions and that this event made the recipient strains antibiotic resistant. The results show that the antibiotic resistance genes present in the general horizontal gene pool can be transferred from environmental strains to pathogenic organisms, but that the transfer ratio is dependent on the recipient strain. The role of natural environments in the evolution of resistance traits in pathogenic bacteria has recently been reviewed (Martinez, 2009). Other studies examined the conjugation between food related (pathogenic) bacteria (Pourshaban *et al.*, 2002; Gevers *et al.*, 2003b; Mc Mahon *et al.*, 2007a; Toomey *et al.*, 2009a), but to our knowledge there are fewer studies describing the transfer from environmental strains to (foodborne) pathogens (Bruun *et al.*, 2003; D'Costa *et al.*, 2006; Walsh *et al.*, 2011).

In this study high transfer ratios were encountered with the highest transfer ratio in the *S. enterica* serovar Enteritidis strain MB 1561 (order of magnitude of 10^{-2}). The plasmid used in this study, pB10, is a broad-host-range plasmid that could be transferred between laboratory strains of *Pseudomonas* sp. and *E. coli*, and from *Pseudomonas* sp. to *Sinorhizobium meliloti* at high transfer ratios with an order of magnitude of 10^{-1} transconjugants per recipient cells (Dröge *et al.*, 2000). Four out of five *E. coli* 0157:H7 recipient strains showed lower transfer ratios than those observed for the *Salmonella* spp. strains. Recently, a study was published describing the dissemination of NDM-1-positive bacteria in the New Delhi environment and its implications for human health (Walsh *et al.*, 2011). NDM-1-positive isolates containing the bla_{NDM-1} gene, were circulating in New Delhi as early as 2006 and plasmids carrying the gene can have up to 14 other antibiotic resistance determinants. These authors found the presence of the bla_{NDM-1} gene in non-fermentative Gram-negative bacteria, like *P. putida*, which were not previously reported to carry this gene. The transfer of bla_{NDM-1} was examined from bacteria, isolated from waste seepage, to the non-pathogenic *E. coli* J53 and to clinical strains of *S. enterica* serovar Enteritidis and *Shigella sonnei*. Transfer into the *S. enterica* serovar Enteritidis and *S. sonnei* recipients was 10 to 1000 times less efficient than into the *E. coli* J53 lab strain. In our study, transfer was more efficient in the *Salmonella* spp. strains than in the *E. coli* strains. It has been demonstrated that the donor affects the host range of pB10 in an activated-sludge microbial community (De Gelder *et al.*, 2005), and it has been posed that in general all conditions

influencing the host, including the genetic background of the host, might also influence the frequency of plasmid transfer by conjugation (Koraimann, 2004).

For all strains, except for *S. enterica* serovar Hadar, transconjugants could be detected by plating and/or by flow cytometry. Other studies also showed that *S. enterica* serovar Hadar is less receptive for mobile genetic elements than other *Salmonella* serovars (Sarowska *et al.*, 2009; Franiczek *et al.*, 2010). It could be that in *S. enterica* serovar Hadar a yet unidentified mechanism hinders the acquisition of plasmid DNA by conjugation. Bacteria have developed defense mechanisms protecting them from invading foreign DNA, some of which directly target the incoming DNA such as restriction-modification systems or CRISPR/Cas systems. In restriction-modification systems a methyltransferase protects host DNA by modifying specific nucleic acids, while the restriction endonuclease cleaves any foreign DNA containing a specific recognition site, which has not previously been protected by the same modification (Dupuis *et al.*, 2013). Although restriction–modification systems act only against double-stranded DNA, it has been demonstrated that the frequency of transconjugants can be reduced if the recipient has a restriction system to which the incoming plasmid is susceptible (Thomas & Nielsen, 2005). However, a strategy to counteract this has been seen in IncP-1 plasmids, from which most restriction–modification sites have been eliminated (Skippington & Ragan, 2011). The clustered regularly interspaced short palindromic repeats (CRISPR), and associated proteins (Cas) system provides bacteria with acquired immunity against viruses and plasmids. In CRISPR loci short, partially palindromic DNA repeats that occur at regular intervals, CRISPR repeats, alternate with variable sequences, CRISPR spacers (Barrangou & Marraffini, 2014). Spacers mostly correspond to segments of captured viral and plasmid sequences (Horvath & Barrangou, 2010). The CRISPR spacers and repeats are transcribed and processed into small CRISPR RNAs (crRNAs) that specify acquired immunity by a sequence-specific mechanism. The prevention of plasmid transfer in *Staphylococcus epidermidis* by this system has been demonstrated by Marraffini & Sontheimer (2008).

Two methods were used in this study for the detection of transconjugants: a cultivation-dependent (plating) and a cultivation-independent method (flow cytometry). The most important advantages of flow cytometry are that it provides a rapid screening of bacterial cultures, takes into account the non-culturable fraction of the bacteria and is less labour intensive than plating. Other studies used flow cytometry in combination with evolutionary algorithms to determine the optimal parameters for transconjugant formation (Boon *et al.*, 2006) or in combination with automated cell sorting of green fluorescent transconjugant cells (Musovic *et al.*, 2006; Shintani *et al.*, 2014). This approach allowed them

to identify the transconjugants (Musovic *et al.*, 2006; Shintani *et al.*, 2014). However, in our study the detection limit was rather high, so rare events could not be observed. For five of the 15 analyzed strains transconjugants could be detected by flow cytometry. With plating, transconjugants were detected for 13 of the 15 analyzed strains. There was one strain (*S. enterica* serovar Enteritidis MB 1139) for which transconjugants only could be detected by flow cytometry and not by plating, even after repeated conjugation experiments. In some cases transconjugants cannot be detected by cultivation because the cells enter into a viable but non-culturable (VBNC) state (Boon *et al.*, 2006). In a previous study, a strain-dependent influence of temperature on the VBNC state was found (Oliver *et al.*, 1995). These authors found a different temperature influence for plasmid-bearing cells and plasmid-free cells of two *Pseudomonas* strains, which was not seen in an *E. coli* strain. Whenever no transconjugants were detected by flow cytometry in our study, the transfer ratios determined by plating were lower than or just around 10^{-5}. These findings indicate that although flow cytometry offers many advantages, it is not always the method of choice due to its high detection limit.

In the last step of this study, the antibiotic resistance profiles of the transconjugants were determined to verify whether the recipient phenotype was altered by receiving the plasmid. Transconjugants were obtained for 13 of the 15 analyzed strains. All these transconjugants showed a decrease in inhibition zone diameter for kanamycin, indicating that they all expressed the kanamycin resistance gene. For the plasmid-encoded antibiotic resistances, the strains showed complete resistance against amoxicillin, sulfonamides and tetracycline. For streptomycin, only slight or no decreases in inhibition zone diameter were observed, resulting in intermediate resistant strains. *E. coli* MB 4021 remained susceptible according to CLSI guidelines although there was a decrease in inhibition zone diameter. Even though there can be a fair to almost perfect agreement between the measurement of minimum inhibitory concentration (MIC) values and the assessment of resistance genes, situations occur in which susceptible isolates carry the corresponding resistance genes (Rosengren *et al.*, 2009). These resistance genes may not be expressed if they are distant from the promoter or if they are associated with a weak promoter in an integron. The same occurs with free gene cassettes which are not incorporated into an integron and lack the integron promoter which is required for expression (Rosengren *et al.*, 2009). An alternative explanation could be a low MIC test sensitivity as is known with *aadA* genes and streptomycin resistance (Rosengren *et al.*, 2009; Sunde & Norström, 2005). A poor agreement was found between genotypes and phenotypes for streptomycin (66% agreement) in a previous study (Boerlin *et al.*, 2005). In the majority

of cases, this disagreement was due to the presence of an *aadA* gene in isolates classified as susceptible to streptomycin. The streptomycin resistance in pB10 is situated on a truncated Tn*5393*c streptomycin resistance transposon. This transposon contains the *strA* and *strB* genes, which encode the two different streptomycin resistance proteins aminoglycoside-3'-phosphotransferase and aminoglycoside-6-phosphotransferase (Schlüter *et al.*, 2003). The association of *strA* and *strB* normally leads to high-level expression of streptomycin resistance (Boerlin *et al.*, 2005; Chiou & Jones, 1995). At the moment, it is not clear to us why the streptomycin resistance was not fully expressed.

5. Conclusion

In this chapter, we demonstrate that an environmental plasmid was transferred to foodborne pathogens (*Salmonella* spp. and *E. coli* O157:H7) under laboratory conditions. The studied recipients contained 10 *Salmonella* isolates belonging to the five serovars which are targeted in the mandatory *Salmonella* control programmes for breeding flocks of *Gallus gallus* according to Commission Regulation (EC) No 1003/2005 (OJEU, 2005a). In these five serovars, and also in a few other serovars, resistance against antibiotics of critical importance in human medicine is observed more frequently than in other serovars (EFSA, 2009). The detection of transconjugants was done by flow cytometry and by plating. Not only does this transfer occur at rather high transfer ratios (up to order of magnitude 10^{-2}), but the acquisition of the plasmid also makes the pathogens resistant to multiple antibiotics. In worst case scenarios, infections with these plasmid-mediated antibiotic resistant pathogens can lead to exacerbation of the patient's condition, treatment failure and thus compromise human health. Therefore, it is important to know if these plasmids can be transferred to potential pathogens and if these antibiotic resistance genes can be expressed in the new hosts.

In the previous chapter, plasmid transfer to foodborne pathogens was investigated. Plasmids can contain integrons which can also contribute to the dissemination of antibiotic resistance. In this chapter a Belgian collection of Shiga toxin-producing E. coli *is screened for the presence of integrons, which are subsequently characterized.*

Chapter 3

Integron characterization and typing of Shiga toxin-producing *Escherichia coli* (STEC) isolates in Belgium

Abstract

The presence of integrons and the antibiotic susceptibility profiles of STEC strains isolated in Belgium were analyzed. The collection contained 306 strains, of which 225 were human isolates and 81 originated from different food or animal sources. Integrons were detected by PCR in 7.5% of the tested isolates and all were class 1 integrons. The integron-positive strains all belonged to the human collection. By RFLP, five different types (A, B, C, D, E) were distinguished. The antibiotic resistance gene cassettes were identified by sequencing representatives of the five different types. Two types of gene cassettes were found in different combinations, one encoding resistance to streptomycin/spectinomycin and the other encoding resistance to trimethoprim. One of the gene cassettes present was the less frequently detected *aadA23*. Susceptibility profiling of the strains for 11 antibiotics was done by standard disk diffusion assays. Among the 23 integron-positive strains, 17 different antibiotic susceptibility profiles were found. In the 283 integron-negative strains, 24 different antibiotic susceptibility profiles were observed. The majority of these strains were susceptible to all tested antibiotics (n = 218, 77.0%). The integron-positive strains were significantly more resistant to eight of the eleven tested antibiotics compared to the integron-negative strains (*P*<0.05). PFGE profiles of integron-positive strains within selected serogroups did not cluster together.

1. Introduction

Besides being a commensal species in humans and animals, *Escherichia coli* also contains strains that can cause disease. These pathogenic strains can be divided into two groups, intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC) (Köhler & Dobrindt, 2011). One subgroup of IPEC are the Shiga toxin-producing *E. coli* (STEC) or synonymously the verocytotoxin-producing or verocytotoxigenic *E. coli* (VTEC). STEC are considered as the most significant group of emerging foodborne pathogens (Bolton, 2011), causing different symptoms, ranging from uncomplicated diarrhoea to very serious illnesses such as haemorrhagic colitis and haemolytic uraemic syndrome (HUS). A few hundred STEC serotypes have been isolated from patients with gastrointestinal disease, but only a few of them seem to be implicated in the majority of human diseases (Karmali *et al.*, 2010).

Although antibiotic treatment remains controversial in the case of a STEC infection (Panos *et al.*, 2006; McGannon *et al.*, 2010), antibiotics are often used in clinical practice. It is important to study how antibiotic resistance genes in STEC can be acquired and possibly be transferred to other bacteria. One kind of genetic element that plays a role in the acquisition and the dissemination of antibiotic resistance genes is the integron. Integrons were first described in the late 1980s (Stokes & Hall, 1989). They are immobile elements that can capture, integrate and express or release gene cassettes. Integrons can be divided into two groups, the mobile integrons, which are associated with mobile DNA elements such as transposons and plasmids, and the chromosomal integrons, which are associated with the bacterial chromosome (Cambray *et al.*, 2010). Based on the sequence of the encoded integrases, five different integron classes can be distinguished among the mobile integrons, of which only the first three are historically associated with the dissemination of multiresistance (Cambray *et al.*, 2010). In Gram-negative pathogens, class 1 integrons are the most abundant, followed by class 2 integrons and the rarely detected class 3 integrons (Stokes & Gillings, 2011). Class 1 integrons usually consist of two conserved segments (5'-CS and 3'-CS) between which one or more gene cassettes can be integrated. In the 5'-CS elements are present for the integration and the expression of the gene cassette(s), namely an *intI1* gene, encoding an integrase, a recombination site *attI* and a common promoter Pc (Sáenz *et al.*, 2010). In the 3'-CS *qacEΔ1* and *sul1* genes are present, encoding resistance to quaternary ammonium compounds and resistance to sulfonamides (Sáenz *et al.*, 2010). A gene cassette consists of a gene and a recombination site, *attC*, by which the cassette can be integrated in

the integron by site-specific recombination. More than 130 gene cassettes associated with antibiotic resistance have been identified in integron classes 1, 2 and 3 (Partridge *et al.*, 2009).

The goal of this study was to screen a large collection of more than 300 STEC strains, isolated between 2000 and 2007 in Belgium from humans, food and animals, in order (1) to investigate the presence of integrons, (2) to characterize the integron-positive strains, (3) to identify the gene cassettes carried by them and (4) to analyze their antibiotic susceptibility. For this purpose multiple techniques (antibiotic susceptibility testing, PCR, RFLP, sequencing and PFGE) were applied.

2. **Material and Methods**

2.1. STEC isolates

A total of 306 strains were investigated in this study. Isolates were collected by the Belgian National Reference Center for VTEC/STEC between 2000 and 2007. The collection screened contained 225 human isolates and 81 strains originating from different food or animal sources. All clinical laboratories in Belgium can submit suspected strains or samples to the reference lab. They are encouraged to send strains or stool samples for all severe cases, in particular HUS. Serogroups were determined by bacterial agglutination using O antisera (Statens Serum Institute, Denmark) for the most prevalent groups. Non-agglutinating isolates were sent to Statens Serum Institute for O:H serotyping.

2.2. Antibiotic susceptibility testing

The susceptibility to 11 antibiotics (ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, trimethoprim, ciprofloxacin, gentamicin, kanamycin, nalidixic acid and cefotaxime) was determined by the disk diffusion method using European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) potency Neo-Sensitabs tablets (Rosco Diagnostica A/S, Denmark). Interpretation of zones was done according to CLSI guidelines, as described by the manufacturer.

2.3. *Presence of the virulence genes*

PCR was used to determine the presence of the following virulence genes: *stx1*, *stx2*, *eae* and *ehx*. Preparation of the DNA samples was done according to the protocol of Flamm *et al.* (1984). The DNA extracts were diluted to a final concentration of 25 ng µL^{-1}. One microlitre was used in the PCRs. Primers (Table 3.1) and PCR conditions were the same as described by Botteldoorn *et al.* (2003). The PCR amplification products were separated by electrophoresis on a 1% Seakem LE agarose gel (Lonza, USA) in 1 × TAE buffer (Invitrogen, USA), visualized by ethidium bromide staining and photographed under UV light.

2.4. *Presence of integrons*

The degenerate primers hep35 and hep36 were used to detect the presence of integrons (Table 3.1). These primers amplify the conserved regions of integrase genes *intI1*, *intI2* and *intI3* (White *et al.*, 2000). The PCR mix contained 1 × buffer, 0.75 mM MgCl$_2$, 1.5 U AmpliTaq DNA polymerase (Applied Biosystems, USA), 50 µM dNTPs, 1 µM of each primer and 2 µL DNA extract (25 ng µL^{-1}) in a total volume of 50 µL. PCR conditions were as described by Nagachinta and Chen (2009). The PCR amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining as described above.

The integron class of the integron-positive samples was determined using integron class-specific primers, being IntI1-F and IntI1-R for integron class 1 and IntI2-F and IntI2-R for integron class 2 (Table 3.1) (Povilonis *et al.*, 2010). The composition of the mix was as mentioned above. The PCR programme for integron class 1 was denaturation for 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 30 s at 68 °C, 1 min at 72 °C, and a final extension for 7 min at 72 °C. The PCR programme for integron class 2 was denaturation for 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 62 °C, 1 min at 72 °C, and a final extension for 8 min at 72 °C. The PCR amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining as described above.

2.5. *Characterization of gene cassette arrays*

Amplification of the gene cassette array of class 1 integrons was done by using the primers 5'CS and 3'CS (Table 3.1) (Povilonis *et al.*, 2010). For this PCR, a long PCR enzyme mix (Fermentas, Lithuania) was used as the length of the fragment was unknown. The mix consisted of 1 × buffer (MgCl$_2$ included), 200 µM dNTPs, 1.5 U Long PCR Enzyme Mix, 1 µM of each primer and 1 µL DNA extract (25 ng µL^{-1}) in a total volume of 50 µL. The PCR

programme was denaturation for 2 min at 94 °C, followed by 30 cycles of 20 s at 94 °C, 30 s at 57 °C, 90 s at 68 °C, and a final extension of 10 min at 68 °C. The PCR amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining as described above.

Further characterization was done by Restriction Fragment Length Polymorphism (RFLP) analysis using the restriction enzymes *Hin*fI (New England Biolabs, USA) and *Rsa*I (GE Healthcare, UK). The restriction mixture for *Hin*fI contained 15 µL amplification product and 0.5 µL restriction enzyme (5 U), the restriction mixture for *Rsa*I contained 15 µL amplification product and 0.5 µL restriction enzyme (3 U). Both restriction mixtures were incubated overnight at 37 °C. The restriction fragments were detected by electrophoresis on a 2% Seakem LE agarose gel in 1 × TAE buffer and visualized by ethidium bromide staining. The different RFLP types were designated with letters (A-E). A selection of amplicons (one per RFLP type) was sequenced by a commercial sequencing facility (Macrogen Inc., Korea). The same primers as for the gene cassette array PCR were used for the sequencing reaction. The sequences were analyzed with Kodon version 3.61 (Applied Maths, Belgium) and comparisons were made using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 3.1. *Primer sequences, target genes, and sizes of amplified PCR products*

Primers	Sequence (5' to 3')[1]	Target gene	Product size (bp)	Reference
VT1-F	ACACTGGATGATCTCAGTGG	*stx1*	614	Botteldoorn et al., 2003
VT1-R	CTGAATCCCCCTCCATTATG			
VT2-F	GGCACTGTCTGAAACTGCTCC	*stx2*	255	Botteldoorn et al., 2003
VT2-R	TCGCCAGTTATCTGACATTCTG			
EAEA-F	GTGGCGAATACTGGCGAGACT	*eae*	890	Botteldoorn et al., 2003
EAEA-R	CCCCATTCTTTTTCACCGTCG			
EHECHLY-F	ACGATGTGGTTTATTCTGGA	*ehx*	165	Botteldoorn et al., 2003
EHECHLY-R	CTTCACGTGACCATACATAT			
hep35	TGCGGGTYAARGATBTKGATTT	*intI1*, *intI2*, *intI3*	491	White et al., 2000
hep36	CARCACATGCGTRTARAT			
IntI1-F	CTGCGTTCGGTCAAGGTTCT	*intI1*	882	Povilonis et al., 2010
IntI1-R	GGAATGGCCGAGCAGATCCT			
IntI2-F	CACGGATATGCGACAAAAAGGT	*intI2*	746	Povilonis et al., 2010
IntI2-R	GTAGCAAACGAGTGACGAAATG			
5'CS	GGCATCCAAGCAGCAAG	Class 1 integron	Variable	Povilonis et al., 2010
3'CS	AAGCAGACTTGACCTGA			

2.6. Pulsed Field Gel Electrophoresis

Strains were characterized by Pulsed Field Gel Electrophoresis (PFGE) according to the rapid protocol of Ribot *et al.* (2006) and as standardized by PulseNet (http://www.pulsenetinternational.org/protocols/). DNA was digested with the restriction enzyme *Xba*I (Roche Diagnostics, Germany) and fragments were analyzed in 1% Seakem Gold agarose gels (Lonza) in 0.5 × Tris-Borate-EDTA (TBE) buffer at 14 °C using the CHEF MAPPER system (Bio-Rad Laboratories, UK). The run time was 19 h at 6 V cm^{-1}, with initial and final switch times of 2.16 and 54.17 s, respectively. Gels were digitally visualized after staining with ethidium bromide, followed by destaining in water. Gel images were analyzed with BioNumerics version 6.5 (Applied Maths). *XbaI*-digested DNA from *Salmonella enterica* subsp. *enterica* serovar Braenderup H9812 was used as the normalization reference. The similarity between PFGE patterns of the same serogroup was calculated using the Dice coefficient (with an optimization of 1.0% and a position tolerance of 1.0%), and the patterns were grouped together according to their similarities using the unweighted pair group method with arithmetic mean (UPGMA) clustering method.

2.7. Statistical analysis

A Chi-square test was performed in Excel 2007 (Microsoft, USA) to assess whether integron-positive strains were significantly more resistant than integron-negative strains for each of the tested antibiotics. For the statistical analysis, intermediate resistant strains were considered as resistant. Significance was set at *P*<0.05.

3. Results

3.1. STEC isolates

A total of 306 STEC strains were investigated. The isolates were classified according their serogroups (Table 3.2). The human strains belonged to more than 25 different serogroups. The most prevalent serogroups among the human strains were O157 (n = 112), O26 (n = 31), O103 (n = 15), O145 (n = 11) and O111 (n = 10). Eighteen serogroups were represented by only one strain. Different clinical manifestations implicated in the human infections were reported, ranging from diarrhoea (23.6%), bloody diarrhoea (21.3%) to HUS (21.8%). In 5.8% of the cases isolates came from patients without diarrhoea, generally in the frame of abdominal pain syndromes. All non-human strains belonged to serogroup O157. Food isolates

accounted for 93.8% of the non-human strains and more than 95% of these were isolated from cattle carcasses, beef and minced beef. The animal isolates came from cattle and from a dog.

3.2. *Antibiotic susceptibility*

For each of the strains, the susceptibility to 11 antibiotics was analyzed and antibiotic susceptibility profiles were determined. In total, 35 different profiles were found. The majority of the strains (71.2%) were susceptible to all the antibiotics tested. This high percentage was caused by the high percentage of susceptible strains (87.0%) within the O157 serogroup. Without taking this serogroup into account, only 44.2% of the strains were susceptible to all the antibiotics tested. In Table 3.2, the number of different antibiotic susceptibility profiles per serogroup is shown. Serogroup O157 was the least diverse serogroup concerning the antibiotic susceptibility profiles, with 11 different profiles among 193 strains. Serogroup O111 was the most diverse group, with 9 different profiles among 10 strains, followed by O145 (7 profiles/11 strains) and O26 (18 profiles/31 strains).

3.3. *Virulence genes*

Isolates were initially screened for the presence of four important STEC-associated virulence genes, *i.e. stx1*, *stx2*, *eae* and *ehx*. STEC strains are characterized by the presence of *stx1* and/or *stx2* genes, which encode Shiga toxins. The *eae* gene encodes intimin, which is associated with adhesion to the intestinal epithelium and the formation of attachment and effacement lesions, and *ehx* is a plasmid-encoded virulence gene encoding enterohaemolysin (Buvens et al, 2012).

Ten different combinations of the four examined virulence genes were found among the screened collection (Table 3.2). The most frequently occurring virulence profile was *stx1⁻*, *stx2⁺*, *eae⁺*, *ehx⁺*, which was present in 56.2% of the strains. Seventy-two of the 306 strains (23.5%) contained the *stx1* gene, 181 (59.2%) contained the *stx2* gene and 53 (17.3%) contained both genes. The *eae* gene was present in 87.9% of the strains, while the *ehx* gene was present in 93.1% of the strains.

Table 3.2. *Overview and characteristics (integron presence, virulence profile and antibiotic resistance profile) of the strains classified per serogroup.*

Serogroup	Number of strains	Integron positive (%)	*stx*1 *stx*2 *eae ehx* profiles (Number)	Number of susceptible strains[§]	Number of different antibiotic resistance profiles
O3	2	2 (100.0)	- + - + (2)	0	2
O5	1	0 (0)	+ + + + (1)	1	1
O6	1	1 (100.0)	+ - - - (1)	0	1
O8	1	0 (0)	+ + - + (1)	0	1
O15	1	0 (0)	+ + - - (1)	1	1
O26	31	6 (19.4)	+ - + - (6) + - + + (22) + + + + (1) - + + + (2)	4	18
O38	1	0 (0)	+ + - + (1)	1	1
O43	1	0 (0)	+ + - - (1)	1	1
O55	1	0 (0)	+ - - - (1)	0	1
O79	1	0 (0)	+ - - - (1)	1	1
O91	2	0 (0)	+ + - + (2)	2	1
O103	15	2 (13.3)	+ - - + (1) + - + + (13) + + + + (1)	7	5
O109/OX182	1[*]	0 (0)	+ - - - (1)	1	1
O111	10	7 (70.0)	+ + + + (4) + - + + (6)	1	9
O113	1	0 (0)	+ + - + (1)	1	1
O117	1	0 (0)	+ - - - (1)	0	1
O118	1	1 (100.0)	+ - + + (1)	0	1
O127	1	0 (0)	+ - + - (1)	0	1
O128ab	2	0 (0)	+ + - + (1) - + - + (1)	2	1
O145	11	2 (18.2)	+ - + + (4) - + + + (7)	3	7
O146	4	1 (25.0)	+ + - + (2) + - - + (1) - + - - (1)	3	2
O153	1	0 (0)	+ - + + (1)	1	1
O156	1	0 (0)	+ - - + (1)	1	1
O157 (Human)	112	1 (0.9)	+ + + + (17) - + + + (95)	98	9
O157 (Food/Animal)	81	0 (0)	+ + + + (14) - + + + (67)	70	6
O166	1	0 (0)	+ + - + (1)	1	1
O175	1	0 (0)	- + - + (1)	1	1
O181	1	0 (0)	+ - - - (1)	0	1
OX182	3	0 (0)	+ - + + (1) + - - + (2)	3	1
O rough	1	0 (0)	- + - - (1)	1	1
Unknown	14	0 (0)	+ + - + (2) + - + + (4) + + - - (2) + - - + (2) - + - + (1) - + - - (2) - + + + (1)	13	2
Total	**306**	**23 (7.5)**	**10[&]**	**218**	**35**

[*] Untypable, cross-reactivity with O109 and OX182 antisera.
[§] Susceptible to all 11 tested antibiotics.
[&] Number of different profiles.

63

3.4. Integron-positive strains

All 306 strains were analyzed for the presence of integrons by PCR. Integrons were detected in 23 strains (7.5%) belonging to nine serogroups (Table 3.2 and Table 3.3). Only class 1 integrons were detected. All four strains belonging to serogroups O3, O6 and O118 possessed an integron. For the other serogroups the highest percentage of integron-positive strains was found in O111 (70.0%), followed by O146 (25.0%), O26 (19.4%) and O145 (18.2%) (Table 3.2). In the O157 serogroup only one integron-positive strain was detected, belonging to the human subgroup.

With the gene cassette array PCR, four different lengths of bands were obtained within these integron-positive strains, except in strain MB 3936 for which no band was detected. The lengths of the bands were visually estimated as ~800 bp, ~1000 bp, ~1600 bp and ~1800 bp. RFLP analysis of these fragments with the restriction enzymes *Hin*fI and *Rsa*I revealed five different types (A, B, C, D, E). The two restriction enzymes revealed the same profile distinction. Fourteen strains (60.9%) belonged to type A and five strains (21.7%) belonged to type C. The other types (B, D and E) were represented by only one strain. By means of sequence analysis the identity of the gene cassette(s) present in the different types was determined. Three types (A, B, E) contained only one gene cassette. Type A and E contained respectively the gene cassette *aadA1* and *aadA23*, encoding resistance to streptomycin/spectinomycin, while type B contained the gene cassette *dfrA7*, encoding resistance to trimethoprim. Although type C and D both contained two gene cassettes, of which one encoded resistance to streptomycin/spectinomycin and the other encoded resistance to trimethoprim, they differed in the antibiotic resistance genes identified. In type C *aadA1* and *dfrA1* were present while in type D *aadA2* and *dfrA12* were detected. The antibiotic resistance profiles of the integron-positive strains are shown in Table 3.3. The strains belonging to type A were all, except one, resistant to streptomycin, as was the case for the strain belonging to type E. The strain of type B (MB 3926) was resistant to trimethoprim. All strains of type C were resistant to streptomycin and trimethoprim while the strain of type D showed intermediate resistance to streptomycin and was completely resistant to trimethoprim.

Table 3.3. *Overview of the integron-positive STEC strains.*

Strain	Integron type	Serogroup	Virulence profile				Antibiotic resistance profile[1]											Clinical manifestation
			*stx*1	*stx*2	*eae*	*ehx*	Str[2]	Tri[3]	Sul	Amp	Chl	Tet	Cip	Gen	Kan	Nal	Cef	
MB 4083	A	O3	-	+	-	+	R	S	R	S	S	S	S	S	R	S	S	Unknown
MB 4243	A	O3	-	+	-	+	R	S	R	S	S	R	S	S	R	R	S	Unknown
MB 3909	A	O26	+	-	+	+	R	S	R	R	R	R	S	S	S	S	S	Diarrhoea
MB 4114	A	O26	+	-	+	+	R	S	R	S	S	S	S	S	S	S	S	Diarrhoea
MB 4126	A	O26	+	-	+	-	R	R	R	R	S	R	S	S	S	S	S	Diarrhoea
MB 4127	A	O26	-	+	+	+	R	S	R	R	S	R	S	S	S	S	S	Unknown
MB 4133	A	O26	+	-	+	-	R	S	R	R	R	R	S	S	S	R	S	Bloody diarrhoea
MB 4119	A	O103	+	-	+	+	R	S	R	R	S	S	S	S	S	S	S	Diarrhoea
MB 4116	A	O111	+	-	+	+	R	S	R	S	S	R	S	S	S	S	S	Diarrhoea
MB 4122	A	O111	+	-	+	+	S	R	R	S	S	R	S	S	S	S	S	Bloody diarrhoea
MB 4239	A	O118	+	-	+	+	R	S	R	S	R	R	S	I	R	R	S	Unknown
MB 3938	A	O145	-	+	+	+	R	R	R	R	R	R	S	S	S	S	S	HUS
MB 4115	A	O145	+	-	+	+	R	S	R	R	R	R	S	S	S	S	S	Unknown
MB 4079	A	O146	-	+	-	-	R	S	R	S	S	S	S	S	S	S	S	Bloody diarrhoea
MB 3926	B	O157	-	+	+	+	R	R	R	R	S	R	S	S	S	S	S	Bloody diarrhoea
MB 4050	C	O6	+	-	-	-	R	R	R	R	S	R	S	S	S	S	S	Bloody diarrhoea
MB 3980	C	O111	+	-	+	+	R	R	R	R	S	R	S	S	S	R	S	Diarrhoea
MB 4030	C	O111	+	+	+	+	R	R	R	R	S	R	S	S	S	R	S	Bloody diarrhoea
MB 4033	C	O111	+	+	+	+	R	R	R	R	S	R	S	S	S	S	S	HUS
MB 4108	C	O111	+	+	+	+	R	R	R	R	S	S	S	S	S	S	S	Bloody diarrhoea
MB 4134	D	O111	+	-	+	+	I	R	S	S	S	R	S	S	S	S	S	Unknown
MB 4131	E	O103	+	-	+	+	R	S	R	R	S	R	S	R	S	S	S	Diarrhoea
MB 3936	No result	O26	+	+	+	+	R	R	R	S	S	R	S	S	S	S	S	HUS

[1]Antibiotic resistance profile was determined for 11 antibiotics: streptomycin (Str), trimethoprim (Tri), sulfonamides (Sul), ampicillin (Amp), chloramphenicol (Chl), tetracycline (Tet), ciprofloxacin (Cip), gentamicin (Gen), kanamycin (Kan), nalidixic acid (Nal) and cefotaxime (Cef).
[2]Streptomycin resistance gene cassettes present in the integrons: type A: *aadA1*; type C: *aadA1*; type D: *aadA2*, type E: *aadA23*.
[3]Trimethoprim resistance gene cassettes present in the integrons: type B: *dfrA7*; type C: *dfrA1*; type D: *dfrA12*.

3.5. Comparison of integron-positive and integron-negative strains

Among the 23 integron-positive strains, 17 different antibiotic susceptibility profiles were found. The profile with resistance to ampicillin, streptomycin, sulfonamides, tetracycline and trimethoprim was the most common (n = 4, 17.4%). In the integron-negative strains, 24 different profiles were observed. The most common among these was the completely susceptible profile (n = 218, 77.0%), followed by the profile with resistances to both streptomycin and sulfonamides (n = 16, 5.7%).

In integron-positive strains, resistance to sulfonamides (95.7%), streptomycin (95.7%), tetracycline (78.3%), ampicillin (60.9%) and trimethoprim (47.8%) was common. When comparing the susceptibilities to these antibiotics, integron-positive strains were significantly more resistant than the integron-negative strains (Table 3.4). Other antibiotic resistances were more restricted, such as resistance to chloramphenicol and kanamycin, which occurred only in some strains of integron type A, while resistance to nalidixic acid occurred in some strains of type A and C (Table 3.3). Integron-positive strains were significantly more resistant to chloramphenicol, gentamicin and nalidixic acid than integron-negative strains. Resistance to ciprofloxacin and cefotaxime was not observed in the integron-positive strains. All integron-negative strains were also susceptible to cefotaxime.

A comparison of PFGE profiles was made between integron-positive and integron-negative strains of serogroups O26, O111 and O145. These serogroups were analyzed because of their relatively high number of integron-positive strains. Among the serogroups studied, clustering of the integron-positive strains was not observed (data not shown).

Table 3.4. *Overview of the antibiotic resistance (%) of the integron-positive and integron-negative strains. Comparison of the resistances between integron-positive and integron-negative strains were done using Chi square tests. The Chi square values and the* P-*values are listed in the table.* P-*values <0.05 were considered to be significant. ND, Not determined.*

Subgroup	Number	Str	Tri	Sul	Amp	Chl	Tet	Cip	Gen	Kan	Nal	Cef
Integron-positive	23	95.7	47.8	95.7	60.9	21.7	78.3	0.0	8.7	13.0	21.7	0.0
Integron-negative	283	19.8	2.5	17.7	7.8	1.1	10.2	0.7	0.4	5.7	1.1	0.0
Total	306	25.5	5.9	23.5	11.8	2.6	15.4	0.7	1.0	6.2	2.6	0.0
Chi square value		64.5	79.0	71.9	57.8	35.7	75.7	0.2	15.2	2.0	35.7	ND
P-**value**		<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.69	<0.05	0.16	<0.05	ND

4. Discussion

Antibiotic resistance is becoming more and more common in STEC (Nagachinta & Chen, 2009, Buvens *et al.*, 2010; Karmali *et al.*, 2010; Cergole-Novella *et al.*, 2011). One type of genetic element by which these organisms can gain and disseminate antibiotic resistance genes is the integron. The screening of a collection of more than 300 STEC strains of human, food and animal origin revealed that integrons were present in 7.5% of the strains. Only integron class 1 was detected. These results are similar to the results of a North American study in which 177 STEC strains were analyzed (Nagachinta and Chen, 2009). The authors found that integron class 1 was present in 7.9% and integron class 2 in 0.6% of the strains. In contrast, Cergole-Novella *et al.* (2011) found a higher percentage (22%) of STEC strains containing integron class 1, isolated from humans and cattle in São Paulo (Brazil). This difference could be due to characteristics of the analyzed collection. They analyzed 32 antibiotic resistant non-O157 STEC strains, while in the study presented here also antibiotic susceptible strains, belonging to O157 and non-O157 serogroups, were analyzed. Taking only the non-O157 serogroups into account in the current study, 19.5% of the analyzed strains were integron-positive. Singh *et al.* (2005) found that 16% of the 274 analyzed STEC strains, isolated in the USA and originating from human patients (n = 81) and sick animals (n = 193; poultry, cattle and swine), contained integron class 1 and in the study of Zhao *et al.* (2001) 18% of the 50 analyzed STEC strains originating from humans, animals and food were integron class 1 positive.

Five different RFLP types could be distinguished among the integron-positive strains. Only for strain MB 3936 the RFLP type could not be determined as there was no amplification of the gene cassette array. It is possible that this strain lacks the 3'-conserved segment preventing one of the primers from annealing, as demonstrated by Sáenz *et al.* (2010). There was not much variation in the antibiotic resistance gene cassettes present in the different types. They all contained genes coding for resistance to streptomycin/spectinomycin (*aadA1*, *aadA2*, *aadA23*) and/or trimethoprim (*dfrA1*, *dfrA7*, *dfrA12*). These results are in accordance with other studies (Cergole-Novella *et al.*, 2011; Povilonis *et al.*, 2010; Skurnik *et al.*, 2005). In the study of Skurnik *et al.* (2005) 85.7% of the class 1 integrons carried *dfr* and/or *aadA* genes. Povilonis *et al.* (2010) reported that the *aadA*- and the *dfr*-containing gene cassettes were the most common in their study, with a frequency of *aadA1* of 60% in the class 1 integron-positive isolates. With its presence in 14 of the 23 integron-positive strains, *aadA1* is also the most common gene cassette array (60.9%) in the current study, followed by the

dfrA1 - aadA1 gene cassette array (21.7%). These two types belong to the most common integron types encountered in *E. coli* isolates, originating from humans and animals in Europe and the USA (Povilonis *et al.*, 2010). The gene cassette present in type E, *aadA23*, is less common. It was first described in a *Salmonella enterica* subsp. *enterica* serovar Agona strain isolated from a pig carcass in Brazil in 2005 (Michael *et al.*, 2005). Thereafter, it has been reported in human *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Salmonella enterica* subsp. *enterica* serovar Bredeney strains in Hungary (Nógrády *et al.*, 2005), in *E. coli* from broilers isolated in the Netherlands in 2004 (van Essen-Zandbergen *et al.*, 2007), in an *E. coli* strain, isolated from neonatal calf diarrhea in Egypt (Ahmed *et al.*, 2009) and in *E. coli* strains isolated from food-producing animals and humans in China (Ho *et al.*, 2009). For 21 of the integron-positive strains, there was a good agreement between the antibiotic resistance phenotype and the gene cassette(s) present. For two strains (MB 4122 and MB 4134) the streptomycin resistance did not come (fully) to expression. This is not surprising, as it is known that the presence of *aadA* gene cassettes in integrons confers low-level streptomycin resistance and therefore represents an obstacle in classifying *E. coli* as susceptible or resistant to streptomycin (Sunde & Norström, 2005).

Most of the integron-positive strains (91.3%) were resistant to at least three different antibiotics. In the integron-negative strains, 13.1% were resistant to at least three antibiotics. Nagachinta and Chen (2009) reported that all integron-positive strains examined in their study were resistant to at least three different antibiotics. The highest resistances among the integron-positive strains were found to sulfonamides (95.7%), streptomycin (95.7%), tetracycline (78.3%), ampicillin (60.9%) and trimethoprim (47.8%). The integron-positive strains were significantly more resistant to these antibiotics than the integron-negative strains. The resistance to sulfonamides, streptomycin and trimethoprim is related to the presence of the integron, while the resistance to tetracycline and ampicillin could be due to the association of mobile integrons with plasmids and transposons (White *et al.*, 2001).

PFGE is used for subtyping of both O157 and non-O157 subgroups and is considered the gold standard of subtyping techniques for epidemiological studies (Karama & Gyles, 2010). In this study, PFGE did not reveal any clustering of the integron-positive strains in the selected serogroups. In the study of Cergole-Novella *et al.* (2011) most of the integron-positive strains, belonging to the O111 serogroup, clustered into two subgroups with more than 90% similarity, while Ho *et al.* (2009) found no clonal relationship (>85% similarity) between human and animal isolates with identical cassettes and also Kang *et al.* (2005) found distinct patterns among *E. coli* isolates carrying identical types of class 1 integrons.

In this study, we report the presence of class 1 integrons in STEC strains circulating in Belgium. The identification of the antibiotic resistance gene cassettes revealed that only two types of antibiotic resistance genes were present in the gene cassettes, but other antibiotic resistances were also present in the integron-positive strains. This is in contrast to the integron-negative strains, of which the majority was susceptible to the tested antibiotics. As integrons are often associated with mobile elements, which can carry additional antibiotic resistance genes, it remains very important to monitor integrons and the antibiotic resistance present in STEC as they can transfer their resistance genes to other (pathogenic) bacteria.

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In Chapter 2, plasmid transfer to foodborne pathogens was investigated. In this chapter, we take one step closer towards the food industry by analyzing plasmid transfer in biofilm models representative for this sector.

Chapter 4

Biofilm models for the food industry:

hotspots for plasmid transfer?

Abstract

Biofilms represent a substantial problem in the food industry, with food spoilage, equipment failure, and public health aspects to consider. Besides, biofilms may be a hotspot for plasmid transfer, by which antibiotic resistance can be disseminated to potential foodborne pathogens. This study investigated biomass and plasmid transfer in dual-species (*P. putida* and *E. coli*) biofilm models relevant to the food industry. Two different configurations (flow-through and drip-flow) and two different inoculation procedures (donor-recipient and recipient-donor) were tested. The drip-flow configuration integrated stainless steel coupons in the setup while the flow-through configuration included a glass flow cell and silicone tubing. The highest biomass density [10 log (cells cm^{-2})] was obtained in the silicone tubing when first the recipient strain was inoculated. High plasmid transfer ratios, up to 1/10 (transconjugants/total bacteria), were found. Depending on the order of inoculation, a difference in transfer efficiency between the biofilm models could be found. The ease by which the multiresistance plasmid was transferred highlights the importance of biofilms in the food industry as hotspots for the acquisition of multiresistance plasmids. This can impede the treatment of foodborne illnesses if pathogens acquire this multiresistance in or from the biofilm.

1. Introduction

Biofilms are the favorable lifestyle of bacteria as they create an advantageous and protective environment. According to Donlan and Costerton (2002), a biofilm is defined as 'a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription'. In food industry, biofilms can become a persisting source of contamination. They can be found everywhere: on the food processing equipment, on the walls or floors of the workspaces, on the walls of storage/transport tanks, or even on the food itself. Both spoilage as pathogenic bacteria can be involved, threatening both the quality of the product as human health. Consequently, this contamination imposes an enormous annual cost of millions (Brooks & Flint, 2008). Concerns for food safety related to biofilms in the food industry have been raised for example in the produce industry (Jahid & Ha, 2012), the dairy industry (Marchand *et al.*, 2012) and the meat industry (Sofos & Geornaras, 2010).

Another important public health aspect associated with the occurrence of biofilms in the food industry is the inherent higher resistance to antimicrobial agents. Several factors can play a role in this feature, such as the matrix, the growth rate, the heterogeneity within the biofilm, the general stress response, quorum sensing, the induction of a biofilm phenotype, and efflux pumps (Mah & O'Toole, 2001; Drenkard, 2003). Besides this inherent resistance, bacteria in biofilms can acquire additional antibiotic resistances from other organisms by horizontal gene transfer. In horizontal gene transfer, three main mechanisms can be distinguished: conjugation (mobile genetic elements are being transferred from a donor to a recipient cell), transformation (uptake of naked DNA), and transduction (bacteriophages as transporters of genetic information). The occurrence of conjugation and transformation in biofilms has been reviewed by Molin and Tolker-Nielsen (2003), and it is becoming more and more clear that both conjugation and transformation are interconnected with biofilm formation (Luo *et al.*, 2005; Reisner *et al.*, 2006; Madsen *et al.*, 2012). The presence of plasmids can positively influence biofilm formation (Ghigo, 2001; Dudley *et al.*, 2006; Burmølle *et al.*, 2008), but it can also have a negative effect as was shown by Røder *et al.* (2013).

As mixed species biofilms are a better representation of biofilms found in the food industry, dual-species biofilms were used in this study. The choice of the bacteria was based on their role in the food industry, namely *Pseudomonas putida* was used as a model for food spoilage organisms, as this environmental species can cause spoilage of, for example, vegetables

(Settanni *et al.*, 2013) and milk (He *et al.*, 2009). *Escherichia coli* was chosen as a model for pathogenic organisms. Although *E. coli* is a commensal species in humans and animals, pathogenic variants, for example Shiga toxin-producing *E. coli* (STEC), exist. Previous studies on different food types such as milk, vegetables, or fish, have also used *P. putida* as an example of spoilage organisms and *E. coli* as an example of pathogenic bacteria (Gunasekera *et al.*, 2002; Feliciano *et al.*, 2010; Settanni *et al.*, 2013).

As biofilms are on one hand an important issue in the food industry and on the other hand ideal environments for horizontal gene transfer, the goal of this study was to (1) quantify dual-species biofilm formation and (2) analyze plasmid transfer in these biofilms. For this purpose, three models were used which differed from each other in the attachment material and in the flow configuration. The attachment material used was stainless steel, silicone, and glass. Stainless steel is a preferred material in the food industry because of its chemical, bacteriological, and organoleptical neutrality, its ease to clean, its durability, and its mechanical characteristics (Zottola & Sasahara, 1994; Marchand *et al.*, 2012). The two biofilm models used are flow displacement models (Coenye & Nelis, 2010). While in the flow-through system, the biofilm is formed under continuous flow conditions with no direct contact with air, the drip-flow system provides an environment for biofilm formation close to the air–liquid interface (Buckingham-Meyer *et al.*, 2007; Goeres *et al.*, 2009). Both models are representative for the food processing environment as the flow-through configuration can be interpreted as model for pipes and tubing, while the drip-flow configuration can stand as a model for conveyor belts or places where drops from leakages hit a metal surface. To our knowledge, it is the first time that a drip-flow reactor in this configuration is used to study plasmid transfer.

2. **Material and Methods**

2.1. Strains, plasmid, and growth conditions

In this study, *P. putida* SM1443 (Christensen *et al.*, 1998), which carried the pB10 plasmid, was used as donor strain, and the laboratory strain *E. coli* DH5α was used as recipient strain. *P. putida* is a strict aerobe bacterium, while *E. coli* is a facultative anaerobe bacterium. The broad-host-range plasmid pB10, belonging to the IncP-1β subgroup, was isolated from a wastewater treatment plant and contains resistance to the antibiotic agents amoxicillin, streptomycin, sulfonamides, and tetracycline and to inorganic mercury ions (Dröge *et al.*,

2000). The plasmid was marked with a *gfp* gene and a kanamycin resistance gene by the insertion of the mini-Tn*5*-Km-P$_{A1-04/03}$::*gfp* cassette (Van Meervenne *et al.*, 2012). The presence of the *gfp* gene enabled the detection of transconjugants by flow cytometry as the donor strain carried the mini-Tn*5*-*lacI*^{q} cassette in its chromosome, preventing the expression of the *gfp* gene in the donor (Christensen *et al.*, 1998).

Preparation of the cultures for inoculating the reactors was the same for the donor and the recipient strain, except that the donor strain was incubated at 28 °C while the recipient strain was incubated at 37 °C, and was standardized as follows: stock cultures of the strains were inoculated on Luria–Bertani (LB) agar plates (10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar per liter). For the donor strain, the LB agar plates contained kanamycin (50 µg mL^{-1}). After overnight incubation, one colony was transferred to 5 mL LB broth. The next day, the OD$_{610 \text{ nm}}$ of this culture was adjusted with LB broth to 0.3, and 2 mL of the adjusted culture was added to 250 mL LB broth. After overnight growth, the culture could be applied to the reactor, having an OD$_{610 \text{ nm}}$ between 0.8 and 1.1 for *P. putida* and between 1.0 and 1.3 for *E. coli* DH5α.

2.2. *Biofilm growth reactor*

To analyze plasmid transfer in biofilms, in-house reactors were built, combining two different flow displacement models, a flow-through system and a drip-flow system (Figure 4.1).

The analyzed flow through system of each reactor, consisted of 4 cm silicone, peroxide cross-linked tubing (VWR International, USA) followed by a glass flow cell consisting of 5.8 - 6.0 cm borosilicate square tubing (Friedrich & Dimmock Inc., USA). Both flow-through systems had an inner diameter of 1 mm. Last in line was the drip-flow system, which was created by enclosing a stainless steel (316L) coupon (7.5 x 2.5 cm) in the reactor on which inocula or media drips. The stainless steel coupons were first cleansed according the protocol used by Speranza *et al.* (2011). The reactors were autoclaved and subsequently placed at an angle of 10° (Goeres *et al.*, 2009).
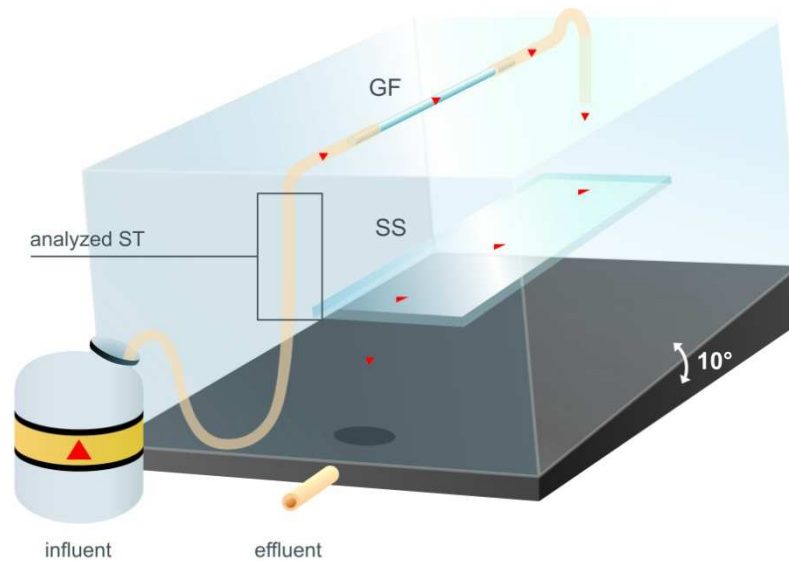
Figure 4.1. *Schematic overview of the in-house reactor. (SS, stainless steel; ST, silicone tubing; GF, glass flow cell).*

2.3. *Biofilm growth and plasmid transfer conditions*

The reactors were placed at 28 °C. In total, eight reactors, containing three types of biofilms, were analyzed. Two inoculation procedures were applied. A first series of four reactors was first inoculated with the donor strain, while the second series of four reactors was first inoculated with the recipient strain. Table 4.1 gives an overview of the different biofilm models. Before the start of the experiment, $0.1 \times$ LB medium was pumped in the reactor for half an hour. Inoculation of the reactors with the first strain was done by pumping an overnight culture during 6 h at a rate of 21–24 mL h^{-1}. This corresponds to a flow rate of 45-51 cm min^{-1} and 35-40 cm min^{-1} for the silicone tubing and the glass flow cell respectively. The rate was kept stable for each reactor, and it corresponded to 15–16 droplets min^{-1} for the drip-flow system. The second strain was provided to the system in the same manner 48 h after the start of the experiment. Between and after the inoculation of the strains, $0.1 \times$ LB medium was applied to the reactor. The reactor was stopped 96 h after the start of the experiment. Subsequently, the stainless steel coupons, the silicone tubing, and the borosilicate tubing were removed for analysis.

Table 4.1. *Overview of the biofilm models.*

Biofilm model	Inoculation strategy	Material	Repeats[1]
DR-SS	Donor-Recipient (DR)	Stainless steel (SS)	4
DR-ST	Donor-Recipient (DR)	Silicone tubing (ST)	4
DR-GF	Donor-Recipient (DR)	Glass flow cell (GF)	3
RD-SS	Recipient-Donor (RD)	Stainless steel (SS)	4
RD-ST	Recipient-Donor (RD)	Silicone tubing (ST)	4
RD-GF	Recipient-Donor (RD)	Glass flow cell (GF)	4

[1] the repeats are biological replicates.

2.4. Biofilm analysis

The stainless steel coupons were washed three times in 0.85% NaCl. The biofilms were removed from the stainless steel with a sterile swab, which was subsequently wringed in a falcon tube containing 10 mL 0.85% NaCl. This was repeated with a second sterile swab. The biofilms attached to the silicone tubing and to the glass flow cell were removed by passing five times 1 mL 0.85% NaCl through each side into a falcon tube. The falcon tubes were vortexed, and biofilms or cell clumps were further mechanically disrupted by pipetting the fluid through a 0.6-mm needle three times.

To analyze the obtained solution, a culture-independent technique, flow cytometry, was chosen as it was not known whether the conditions encountered by the bacteria in the reactor would be able to induce the viable but non-culturable (VBNC) status. By flow cytometry, the total biomass (cells cm^{-2}) and transfer ratio (number of transconjugants/total cell count) were determined. Detection and quantification of (transconjugant) bacteria by flow cytometry were performed with a Cyan ADP Flow Cytometer (Dako, Denmark), using the 488-nm laser. The dilution factor was visually assessed and ranged from 10 to 1000. Dilutions were made with filter-sterilized Evian water. Samples were analyzed without and with a live/dead staining. The staining solution contained propidium iodide and SYBR$^{®}$ Green I, and it was prepared as described by De Roy *et al.* (2012). The unstained samples consisted of 980 µL of the diluted sample, 10 µL Na_2EDTA (500 mM, pH 8), and 10 µL Dako Cytocount beads, while the stained samples consisted of 970 µL of the diluted sample, 10 µL Na_2EDTA (500 mM, pH 8), 10 µL live/dead staining, and 10 µL Dako Cytocount beads. The beads were used to

determine the cell concentration. Green fluorescence emission was collected with a photomultiplier tube using a 530/40 emission filter, for PE 585 and 670 fluorescence a 575/25 and 680/30 emission filter was used, and side light scatter (SSC) was collected using a 488/10 emission filter. The sheath fluid consisted of Milli-Q water. The threshold trigger was set to SSC for the unstained samples and to green fluorescence for the stained samples. The analysis of a sample was performed by collecting data for 1 min in threefold. SUMMIT v4.3 software was used to process the results. By analysis of the unstained samples, the number of transconjugant cells could be determined on a Green Log vs. PE 585 Log plot, while analysis of the stained samples determined the total cell count using a Green Log vs. 670 Log plot. The total cell count equals the sum of the live and dead cell counts.

2.5. *Filter mating*

In previous experiments, plasmid transfer between *E. coli* DH5α and *P. putida* SM1443 (pB10::*gfp*) was studied by filter mating. The filter mating and analysis by flow cytometry were performed as described by Van Meervenne *et al.* (2012).

2.6. *Denaturing gradient gel electrophoresis*

Denaturing gradient gel electrophoresis (DGGE) was used to exclude contamination of the cultures or the biofilms and to obtain an idea of the relative abundance of *P. putida* and *E. coli* in each biofilm. Therefore, both donor and recipient cultures as samples from the different biofilms were analyzed by DGGE. DNA extractions were performed according to Boon *et al.* (2000). DGGE was applied to separate PCR products of 16S rRNA genes obtained with general bacterial primers (338F-GC and 518R) (Muyzer *et al.*, 1993). The PCR products were loaded onto 8% polyacrylamide gels with a denaturing gradient from 45% to 60%. The gels were run on an Ingeny PhorU-2 × 2 apparatus (Ingeny International, The Netherlands). Analysis was carried out using BIONUMERICS software version 5.10 (Applied Math, Belgium). Previous studies have evaluated the potential of DGGE as a semi-quantitative tool (Riemann *et al.*, 1999; Casamayor *et al.*, 2000; Schauer *et al.*, 2000; Lyautey *et al.*, 2005). To assess the relative abundance of *P. putida* and *E. coli*, the ratio of the peak height for one of each strains to the sum of the peak heights for *P. putida* and *E. coli* was determined.

2.7. *Statistical analysis*

All statistical analyses were performed in R. The significance level was set at 0.05. Normality of the residuals was studied by means of QQ-plotting and the Kolmogorov-Smirnov test. Biomass density, transconjugant density, and plasmid transfer ratio were log-transformed so that normality of the residuals was respected. Homoscedasticity of the variances was assessed using the modified Levene's test. Significant differences were detected using one-way ANOVA followed by post hoc analysis according to Tukey.

Outliers were detected by calculating studentized residuals. Their impact on the outcome of the statistical model was evaluated using DfFITS and Cook's distance.

3. Results and Discussion

We analyzed the potential of plasmid transfer among bacteria growing in biofilms, formed in model systems that are representative for food industry (Figure 4.1). The models were placed in a serial order with the last one being the drip-flow system, in which a preferred material of the food industry, namely stainless steel, was used. In the flow-through system, the glass flow cell was chosen as this setup is regularly used in biofilm studies. For the third model, a flow-through system as well, a material was used that was air permeable and that could be found in the food industry, namely silicone. For these three different attachment materials, two inoculation procedures were applied (Table 4.1). A first inoculation procedure involved the formation of plasmid-donating biofilms and subsequent inoculation with a recipient strain. In the second inoculation procedure, biofilms were formed with a plasmid-receiving strain upon which the donor strain was added.

3.1. *Biofilm biomass*

To compare the biomass obtained in the different biofilm models, biomass density was calculated as the log number of cells per cm^2 (Figure 4.2). Enumeration of the cells was done by flow cytometry. For the first inoculation procedure (donor–recipient), the stainless steel model yielded a slightly higher biomass density [8.80 ± 0.17 log (cells cm^{-2})] than the silicone tubing model [8.20 ± 0.38 log (cells cm^{-2}), $P = 0.01$] and the glass flow model [8.30 ± 0.24 log (cells cm^{-2}), $P = 0.08$]. For the second inoculation procedure (recipient-donor), a remarkable increase in average biomass density was found in the silicone tubing model [10.23 ± 0.05 log (cells cm^{-2})]. One of four replicates of the former model

yielded outlying results (Figure 4.2). The reason for this outlier is unclear to us. The reactors were composed and run pairwise under the same conditions. Furthermore, the $OD_{610\,nm}$ of the inocula was comparable for both the recipient as the donor strain (data not shown). Based on the DfFITS and Cook's distance analyses, it was concluded that the outlier had a strong influence on the outcome of the statistical model, and it was therefore decided not to include this value.
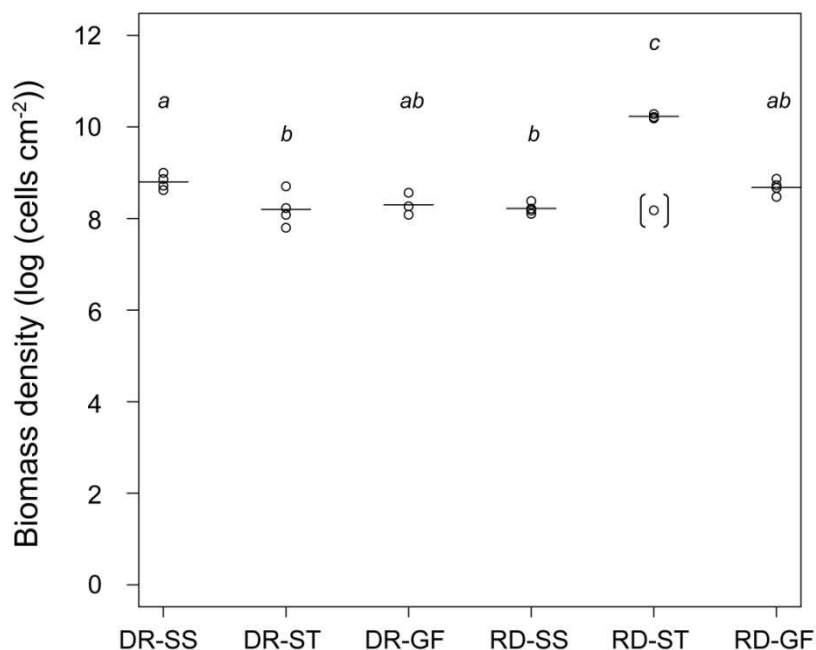

Figure 4.2. *Biomass density for the different biofilm models. See Table 4.1 for the abbreviations. Circles represent the separate values of the replicates, while the line represents the average of the model. The circle between the brackets represents the outlier, which was not included in the analysis. Mean values sharing common lowercase letters are not significantly different (*P < 0.05*).*

It is difficult to explain why the highest biomass density was obtained in the silicone tubing with the second inoculation procedure. Bacterial attachment is influenced by a variety of factors, including bacterial features (*e.g.* bacterial hydrophobicity, cellular surface charge, surface structures, and outer membrane proteins), but also features of the used material (*e.g.* chemical composition, surface roughness, hydrophobicity) and features of the surrounding environment (Goulter *et al.*, 2009; Shi & Zhu, 2009). Previous studies have found, for example, that anaerobic conditions inhibit *E. coli* biofilm formation (Colón-González *et al.*, 2004; Cabellos-Avelar *et al.*, 2006); however, in biofilm flow cells, traces of oxygen are

expected, and the silicone tubing used is air permeable. Another factor that can influence the bacterial attachment is the hydrophobicity of the material. Both the silicone tubing and the stainless steel are hydrophobic material, while glass is hydrophilic. Andersen *et al.* (2010) showed using uropathogenic *E. coli* that the influence of the hydrophobicity of the contact material is not species-dependent but rather isolate-dependent.

Interestingly, DGGE analysis indicated that the relative abundance of *E. coli* DH5α was much higher in both flow-through systems when the recipient strain was inoculated before the donor strain (Figure 4.3). In the other biofilm models, a comparable relative abundance was found for both *E. coli* and *P. putida*. The lack of oxygen may possibly play a role herein. Very few studies which worked with *E. coli* and *P. putida* biofilms looked at the abundances of the strains. Castonguay *et al.* (2006) found equal concentrations of *E. coli* and *P. putida* in mixed biofilms, formed in glass tubes, while Gilbert *et al.* (2003) found a significantly higher proportion of *P. putida* in mixed biofilms formed in flow cells, which was attributed to the different ability to adhere of the two strains. However, these studies were not conducted with the same experimental design, which complicates the comparison of results. The process that was mimicked in our experiments was co-adhesion, which means that planktonic cells adhere to biofilm cells (Bos *et al.*, 1994; Rickard *et al.*, 2003), while in the two mentioned studies, the inoculum was mixed.

Overall, the results indicate that for the biomass, it is difficult to assign determining factors, but it seems that order of inoculation and attachment material rather than flow configuration may play a role. Considering the relative abundances of *E. coli* DH5α and *P. putida* SM1443 (pB10:: *gfp*), it appears that depending on the order of inoculation, the flow configuration can have an influence.
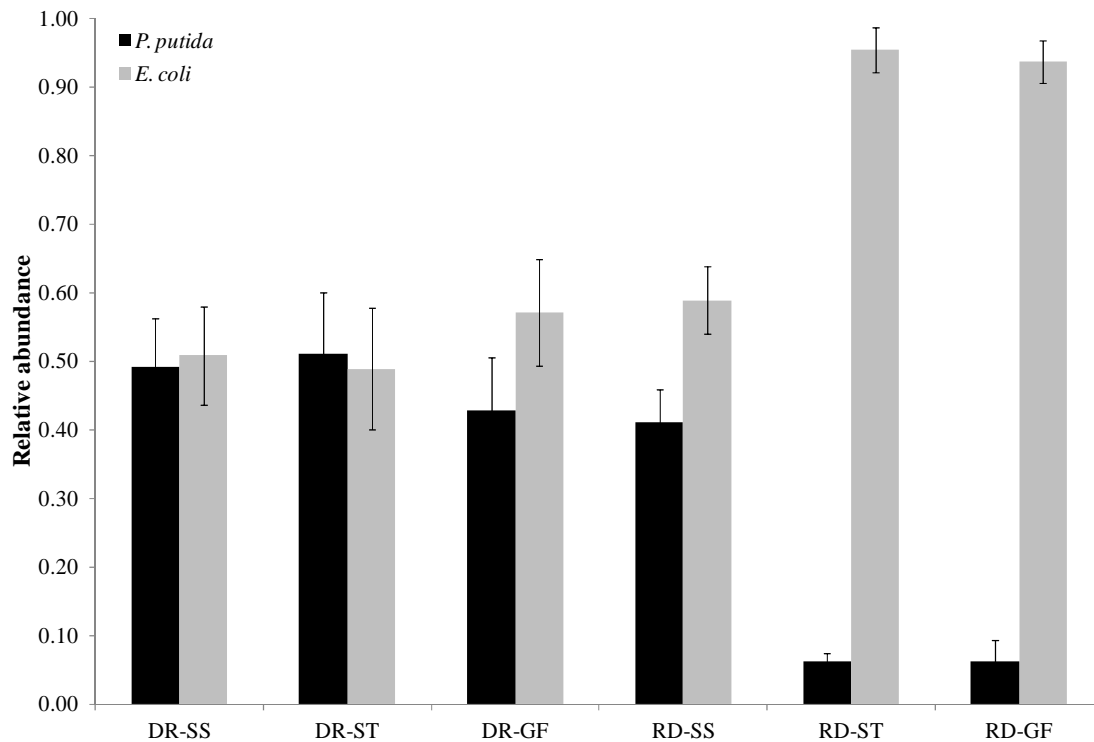
Figure 4.3. *Estimated relative frequency of* P. putida *(black bars) and* E. coli *(grey bars) in the different biofilm models (n = 4, for DR-GF n = 3) determined by DGGE. See Table 4.1 for the abbreviations.*

3.2. Plasmid transfer

In the present study, plasmid transfer ratio was expressed as the log of the ratio of the number of transconjugants to the total cell count. Table 4.2 shows the number of transconjugants for each replicate of the different models. In previous experiments, the transfer ratio was determined for filter mating. In those experiments, an average transfer ratio of 1/100 was obtained (data not shown). In the biofilm models, the average transfer ratio for the first inoculation procedure (donor–recipient) ranged between 2/100 and 1/10, and no significant difference was found between the three models (Figure 4.4). With the other inoculation procedure (recipient–donor), the average transfer ratio ranged between 5/10 000 and 8/100. The transfer ratio obtained for the three models using this inoculation procedure differed significantly from each other. Furthermore, the RD-ST model differed significantly from the DR-ST model, indicating an influence of the order of inoculation involved. As donor and recipient strain are not the same strain with or without the plasmid, it is difficult to obtain indications about the influence of the plasmid.

Table 4.2. *Number of transconjugants, expressed as log (cells cm^{-2}) for each replicate of the different models. The value between brackets represents the outlier as determined by the statistical analysis of the biomass.*

	DR-SS	DR-ST	DR-GF	RD-SS	RD-ST	RD-GF
Reactor 1	7.25	7.17	6.81	7.30	(6.52)	6.45
Reactor 2	7.00	7.50	6.08	6.93	7.14	6.37
Reactor 3	7.34	6.62	6.80	7.15	6.77	6.12
Reactor 4	6.95	6.67	-	7.00	6.91	6.52
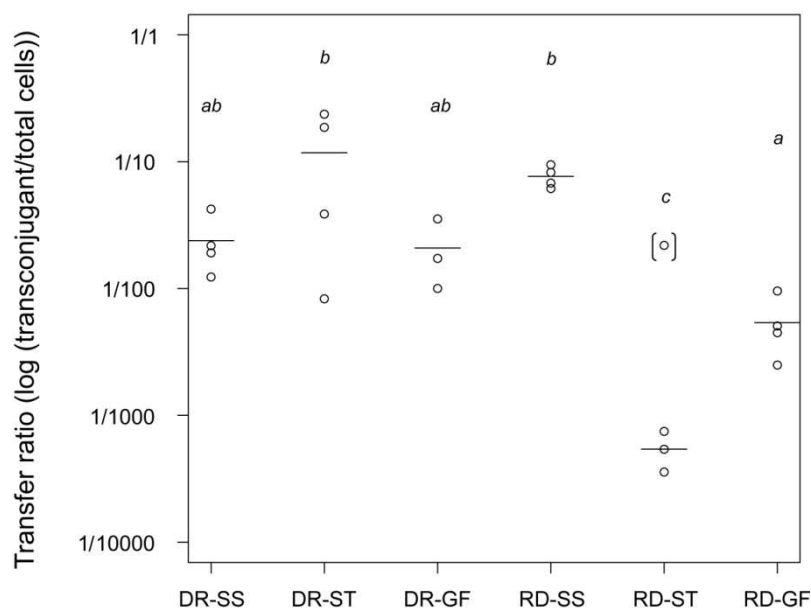
Figure 4.4. *Transfer ratio of the different biofilm models. See Table 4.1 for the abbreviations. Circles represent the separate values of the replicates, while the line represents the average of the model. The circle between the brackets represents the outlier, which was not included in the analysis. Mean values sharing common lowercase letters are not significantly different (P < 0.05).*

Biofilms are considered to be hotspots for plasmid transfer. Several studies have found higher transfer frequencies under biofilm conditions than with planktonic cultures (Lampkowska *et al.*, 2008; Nguyen *et al.*, 2010; Hennequin *et al.*, 2012; Savage *et al.*, 2013). The plasmid used in this study, pB10, belongs to the incompatibility group IncP-1β. The IncP-1 plasmids, which are known to encode short rigid pili, transfer best in surface matings (Bradley *et al.*, 1980; Bradley, 1983). Comparing the obtained transfer results with the results of previous studies is difficult due to differences in experimental design, such as reactor design, used strains and plasmids, inoculation methods, detection methods, etc. For instance, De Gelder *et al.* (2005)

found that, when using the same plasmid (pB10, *rfp* labeled), the diversity of transconjugants depended on the chosen donor and on the mating type. Furthermore, Lilley and Bailey (2002) showed that the recipient had a significant influence on the transfer efficiency of pQBR11-V1 from *P. putida*. In both studies, the same donor strain as in this study was used. High transfer ratios were obtained in the present study, both for the flow-through as for the drip-flow conditions. This is in contrast to the study of Król *et al.* (2011) who found a very low occurrence of plasmid transfer in submerged biofilms formed in closed horizontal flow cells under different conditions, but a large number of transconjugants in a biofilm formed at the air–liquid interface. An explanation for this could be the observed spatial separation of donor and recipient cells as for the biofilm formation of the strains they used (*E. coli* K-12), the presence of conjugative plasmids (in this case pB10) was required. Furthermore, these authors found that there was no statistically significant difference in conjugation efficiency between aerobic and anaerobic matings with aerobically grown donor and recipient cultures. Nevertheless, our study also indicates that the air–liquid interface can be a place of preference for plasmid transfer.

In our experiments, no clear link between transfer ratio and surface hydrophobicity could be observed. In a previous study in which plasmid transfer was analyzed in biofilms formed on hydrophilic and hydrophobic glass beads, a more efficient transfer was observed on the hydrophilic surface (Angles *et al.*, 1993). A possible explanation for this was the difference in biofilm structure as it appeared that morphological changes were induced in the marine bacterium that was used in this study. On the hydrophobic surface, tightly packed biofilms were formed while on the hydrophilic surface, tangled filaments were formed that could possibly trap more donor cells resulting in greater gene transfer frequencies (Dalton *et al.*, 1994).

In conclusion, the obtained results suggest that depending on the order of inoculation, an effect of biofilm model on plasmid transfer ratio can occur. Furthermore, this study also demonstrated that the drip-flow configuration can be used to study plasmid transfer.

The threat that the presence of antibiotic resistance in the food industry poses on human health has recently been reviewed (Capita & Alonso-Calleja, 2013; Verraes *et al.*, 2013). Using two different flow configurations and three different attachment materials, it was shown that (1) biofilms were easily obtained in models relevant to the food industry and (2) a multiresistance plasmid could easily be transferred in the different biofilm models. Together,

these results highlight the importance of biofilms in the food industry as hotspots for the acquisition of multiresistance plasmids next to their obvious contamination potential.

4. Acknowledgments

The previous chapters have dealt with antibiotic resistance in Gram-negative bacteria. It was found that Gram-negative foodborne pathogens can acquire antibiotic resistance and that the obtained transconjugants express the acquired resistance genes. Subsequently plasmid transfer was demonstrated in biofilm models representative for the food industry. In this chapter the focus is on the influence of food preservation on plasmid transfer using a Gram-positive model.

Chapter 5

Low temperature and modified atmosphere: Hurdles for antibiotic resistance transfer?

Abstract

It is acknowledged that food is an important route by which antibiotic resistant bacteria can disseminate. However, there is a lack of knowledge about how factors, which are used during food production and preservation, contribute to the transfer of antibiotic resistance genes.

In this study, the effect of two important techniques widely applied in food preservation, low temperature and modified atmosphere packaging (MAP), on antibiotic resistance transfer have been evaluated. Filter mating experiments with high density inocula were conducted on non-selective agar plates to analyze the temperature range in which an antibiotic resistance plasmid is transferred from *Lb. sakei* subsp. *sakei* to *L. monocytogenes* and to assess the effect of three atmospheres (air, 50% CO_2/50% N_2 and 100% N_2) on the occurrence of plasmid transfer. MAP experiments were subsequently performed on slices of cooked ham, first with high density inocula and afterwards with low density inocula to approach more realistic conditions.

In the temperature experiment, plasmid transfer was observed between 10 °C and 37 °C. The lower limit could be decreased when the incubation period was prolonged. When high density inocula were used, transconjugants were detected, both on agar plates and cooked ham, under the three atmospheres at 7 °C yielding an average transfer ratio (number of transconjugants/number of recipients) with an order of magnitude of $10^{-4} – 10^{-6}$. In the more realistic set-up, with low density inocula, transfer was only detected under the 100% N_2 atmosphere after an incubation period of 10 days at 7 °C, yielding a transfer ratio of 10^{-5}. Under this condition the highest bacterial density was obtained.

Overall, it seems that low temperature and MAP, two important hurdles preventing bacterial growth in the food industry, do not necessarily prevent plasmid transfer to occur.

1. Introduction

The food chain is an important source of antibiotic resistance. Contamination of food with antibiotic resistant bacteria can occur in several ways: I) the use of antibiotics in the primary production exerts a selective pressure towards antibiotic resistant bacteria, which can contaminate the primary food product, II) bacteria that are intentionally added to the food can be vectors for the transfer of antibiotic resistance, III) in every step of food production contamination with antibiotic resistant bacteria is possible (Verraes *et al.*, 2013).

To meet the consumer demand for safe and high-quality food, the food industry has switched increasingly to minimal processing techniques. Minimal processing involves processing methods that change the inherent fresh-like quality characteristics of the food as little as possible (minimally) but at the same time provide the food product with a sufficient shelf life (Ohlsson, 1994). The stress imposed on bacteria during minimal processing can however influence the expression of antibiotic resistance (phenotypic antibiotic resistance). Both increases as decreases in phenotypic antibiotic resistance have been observed. McMahon *et al.* (2007b) observed a decreased phenotypic antibiotic resistance under temperature stress, while an increased phenotypic antibiotic resistance was observed under acid and salt stress for *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus*. They also demonstrated that in some cases the change in phenotypic antibiotic resistance was maintained even after removal of the food preservation stress (McMahon *et al.*, 2007b). Ganjian *et al.* (2012) found that salt stress significantly increased antibiotic resistance to rifampicin, penicillin and methicillin in *S. aureus*, while for gentamicin a small decrease in antibiotic resistance was observed. Al-Nabulsi *et al.* (2011) analyzed the effect of heat, cold, extreme pH conditions and desiccation on the phenotypic antibiotic resistance of *Cronobacter sakazakii* to 13 antibiotics. Different responses were observed depending on the stress/antibiotic combination, with also strain dependent effects. Another important aspect to be considered is the fact that some stress parameters which are used during food production and preservation (such as low temperature, reduced pH, increased osmotic stress) can have an influence on the rate of plasmid transfer and hence on the transfer of plasmid located antibiotic resistance genes. The influence of biotic and abiotic factors on plasmid transfer, has indeed been observed in environmental microbiological studies (reviewed by van Elsas & Bailey, 2002). This has been less extensively analyzed in food processing related studies. The studies performed indicated that the sublethal stresses (*e.g.* temperature, pH, salt) imposed on food pathogens in modern food preservation systems

can have an increasing effect on conjugation rates (Beuls *et al.*, 2012; Toomey *et al.*, 2009b; Walsh *et al.*, 2008; Mc Mahon *et al.*, 2007a).

One important technique applied in minimal processing is modified atmosphere packaging (MAP). Nowadays, it often forms an essential hurdle to guarantee an acceptable microbial shelf life. In MAP, the air in the package is replaced by a specific mixture of gases of which carbon dioxide (CO_2), nitrogen (N_2) and oxygen (O_2) are the most frequently applied (Farber, 1991). Although each gas has its specific function, it is especially CO_2 that has antimicrobial effects and it is most effective in foods where aerobic, Gram-negative psychrotrophic bacteria constitute the normal spoilage community (Philips, 1996). Some Gram-positive spoilage bacteria such as *Lactobacillus* spp. or *Brochotrix thermosphacta* are usually resistant against inhibition by CO_2 (Farber, 1991). As the solubility of CO_2 decreases with increasing temperatures, it is important to respect the low storage temperatures (Farber, 1991). At cold temperatures only psychrotrophic and psychrophilic bacteria are able to grow. One of the few bacterial pathogens that can multiply at low temperatures is *Listeria monocytogenes*. The major public health concern related to this pathogen is its high mortality rate. Recent surveillance data indicated case fatality rates of 12.7% and 21% (EFSA/ECDC, 2013; Silk *et al.*, 2013). Ready-to-eat (RTE) foods with a prolonged shelf life and stored under refrigerated conditions, are considered to be risk products for listeriosis (Uyttendaele *et al.*, 2009). A recent European Union level survey on three types of RTE food showed a prevalence of *L. monocytogenes* of 10.3% in fish, 2.07% in meat and 0.47% in cheese, while the percentages exceeding the level of 100 CFU g^{-1} at the end of shelf life amounted to 1.7%, 0.43% and 0.06% for fish, meat and cheese samples, respectively (EFSA, 2013).

The aim of this study was to 1) investigate the temperature range in which plasmid transfer from *Lactobacillus sakei* subsp. *sakei* to *L. monocytogenes* occurs, and 2) evaluate the effect of atmosphere on plasmid transfer as it is currently unknown if MAP can have an influence on plasmid transfer. For this purpose, *Lb. sakei* subsp. *sakei* was used as donor strain as this is a typical Gram-positive spoilage bacterium and *L. monocytogenes* was used as recipient strain representing a Gram-positive psychrotrophic pathogen. Furthermore, these two species can be found on RTE food packaged under modified atmosphere. The experiments analyzing plasmid transfer under MAP conditions were conducted on non-selective agar plates and on slices of cooked ham. Experiments were first conducted with high density inocula to verify the possibility of occurrence of plasmid transfer under optimal conditions and subsequently with low density inocula on the slices of cooked ham to mimic more realistic conditions.

2. Material and Methods

2.1. Bacterial strains and preparation of bacterial stocks

The donor strain, *Lb. sakei* subsp. *sakei* (LMG 21682), was obtained from the BCCM/LMG public collection. This strain carries a plasmid-encoded *tet*(M) gene and was originally isolated from fermented dry sausage (Gevers *et al.*, 2003a). The plasmid had a size of approximately 10 kb and the *tet*(M) gene was not located on a transposon of the Tn*916*/Tn*1545* family (Gevers *et al.*, 2003a). Partial sequencing of the *tet*(M) open reading frame (GenBank accession number AY149584) indicated high sequence similarities (>99.6%) with *tet*(M) genes previously reported in *Neisseria meningitidis* (Gevers *et al.*, 2003a). The recipient strain, *L. monocytogenes* 4b (MB 4572), was isolated from meat. The recipient strain was made resistant to rifampicin by reculturing it daily on Tryptone Soya Agar (TSA) plates containing a doublefold rifampicin concentration. Bacterial stock cultures stored at -80 °C were used to prepare the inocula for the filter mating experiments. These stock cultures were made by diluting a fresh liquid culture in de Man, Rogosa and Sharpe (MRS) broth for the donor strain and in Brain Heart Infusion (BHI) broth for the recipient strain and subsequently incubating at 37 °C (aerobically for the recipient strain and anaerobically for the donor strain). Bacteria were collected at an optical density at 600 nm ($OD_{600\,nm}$) of 1.0 for the donor strain and 0.75 for the recipient strain. Subsequently, 15% (v/v %) glycerol was added and aliquots were stored at -80 °C.

2.2. Filter mating

Plasmid transfer experiments were conducted on sterile 0.45 μm mixed cellulose esters filters (25 mm diameter) (Millipore, USA). For each experiment, frozen aliquots of the stock cultures of the donor and recipient strain were left 5 minutes at room temperature before adding 200 μL of these to 5 mL of MRS broth for the donor strain and of TSB containing 0.6% yeast extract (TSBYE) for the recipient strain. After 3.5 hours incubation at 37 °C under microaerophilic (5% O_2) conditions (donor strain) and aerobic conditions (recipient strain), the strains were diluted. Subsequently, 1 mL of the donor strain was mixed with 1 mL of the recipient strain. This mixture was applied on the filters using a Swinnex filter holder (Millipore). The inoculated filters were subsequently put on the appropriate medium and incubated at the conditions indicated below. After incubation, the filters were transferred into 2 mL Ringer solution (Oxoid) and were vortexed twice during 1 minute (wash solution). The suspended bacteria were analyzed by plate counting of serial dilutions. For the detection of

transconjugants TSAYE plates containing 50 µg mL^{-1} rifampicin and 10 µg mL^{-1} tetracycline were used. The enumeration of the donor strain was done on MRS plates containing 10 µg mL^{-1} tetracycline, while for the enumeration of the recipient strain TSAYE plates containing 50 µg mL^{-1} rifampicin were used. The plates selective for the transconjugants and the recipient strain were incubated for 48 hours at 37 °C under aerobic conditions, while the plates selective for the donor strain were incubated for 48 hours at 37 °C under microaerophilic conditions.

The transfer ratio was determined as ratio of the number of transconjugants to the number of recipients.

2.3. Influence of temperature

To analyze the influence of temperature on plasmid transfer, both cultures of donor and recipient were diluted 100 times in the suitable liquid growth medium before applying on the filter. The filters were subsequently put on TSAYE plates. These plates were incubated at 7 °C, 10 °C, 15 °C, 22 °C and 37 °C. After an incubation period of 20 hours, the filters were washed and analyzed. For each temperature 4 filters were analyzed.

2.4. Influence of modified atmosphere packaging

To determine the influence of modified atmosphere packaging on plasmid transfer, three different experiments were conducted in which each time three different atmospheric conditions were tested: air, 50% CO_2/50% N_2 and 100% N_2. The packaging under modified atmosphere was done as follows: the plates containing the filters (n = 3) were attached in polypropylene trays. These trays were sealed after adding the right gas mixture with a PET/CPP NPAF foil using a tray sealing machine (vc999, Switzerland). The foil is 62 µm thick and has an oxygen permeability of 190 cm^3 m^{-2} 24h^{-1} (25 °C, 50% R.H.). In the first experiment (Agar_High density), filters were prepared as described above and subsequently put on TSAYE plates. After packaging, the trays were incubated at 7 °C and the filters were analyzed after 5 and 10 days. Before analyzing the filters, the gas composition in the trays was determined using a headspace gas analyzer (PBI-Dansensor A/S, Denmark). In the second experiment (Ham_High density), the filters were put on slices of cooked ham (6.5 ± 0.4 g), which were put into Petri dishes. The cooked ham was bought in a Belgian supermarket. Both physicochemical and microbiological parameters of the cooked ham were determined by different labs of the Technology and Food Science Unit of ILVO (Table 5.1).

The preparation of the filters was done as in the first MAP experiment, except that in this experiment both donor and recipient strains were diluted in maximum recovery diluent (MRD) (Oxoid) instead of liquid growth medium. After packaging, the trays were incubated at 7 °C during 5 days. The third experiment (Ham_Low density) was conducted in the same manner as the second experiment, with the only exception that the filters were inoculated with a lower density of donor and recipient strain as this represents a more realistic situation. The donor and recipient strains were respectively 10^6 and 10^7 times diluted in MRD before adding on the filters. Filters were analyzed after 5 and 10 days. In this last experiment tenfold dilutions of the wash solution were made for the enumeration of donor and recipient bacteria, but for the detection of transconjugants the remaining wash solution was plated totally on double selective medium.

Table 5.1. Range of physicochemical and microbiological parameters of the cooked ham used in the different experiments.

Parameter	High density experiment	Low density experiment
pH	6.01 - 6.15	6.10 - 6.17
a_w	0.976 - 0.978	0.973 - 0.974
Salt (%)	1.9 - 2.1	1.1 - 1.2
Moisture content (%)	74.1 - 74.6	73.4 - 73.6
Lactate (%)	0.709 - 0.785	0.629 - 0.655
Acetate (%)	0.059 - 0.135	0.006 - 0.009
L. monocytogenes	Absent	Absent
Lactic acid bacteria (CFU g^{-1})	8.5×10^2 - $>3.0 \times 10^5$	< 10.0
Total psychrotrophic number (Aerobic) (CFU g^{-1})	1.3×10^3 - $>3.0 \times 10^5$	< 10.0
Total psychrotrophic number (Anaerobic) (CFU g^{-1})	1.1×10^3 - $>3.0 \times 10^5$	< 10.0

2.5. Confirmation of the transconjugant status

To confirm that the colonies detected on the double selective plates were indeed *L. monocytogenes* containing the plasmid, a selection of putative transconjugants were picked from the double selective plates and plated onto new double selective plates. After overnight incubation at 37 °C under aerobic conditions, lysates were made by adding a full inoculation loop to 100 µL sterile water and boiling this for 10 minutes. The lysates were stored at -20 °C. For the specific detection of *L. monocytogenes*, a PCR was performed with the primers

Lm486-505F (5'-ACAAGCTGCACCTGTTGCAG-3') and Lm1060-1079R (5'-GAACCTTGATTAGCATTCGT-3') as described by Van Coillie *et al.* (2004). A second PCR detected the presence of the *tet*(M) gene using the primers *tet*(M)-F (5'-GTGGACAAAGGTACAACGAG-3') and *tet*(M)-R (5'-CGGTAAAGTTCGTCA CACAC-3') according to the thermal cycling protocol of Toomey *et al.* (2009a).

2.6. Statistical analysis

Before analysis, normality was tested by the Shapiro-Wilk test. Because normality could not be obtained for every situation, not even after transformation of the data and because the small number of repeats, it was decided to perform non-parametric tests using the Kruskal-Wallis test. Analyses were performed using IBM SPSS 22.0 (IBM Corp., USA) and significant differences were considered at $P < 0.05$.

3. Results

The filter mating technique was used to study the transfer of an antibiotic resistance plasmid from *Lb. sakei* subsp. *sakei* to *L. monocytogenes* under several food related stress factors. In each experiment the transfer ratio is expressed as the log of the ratio of the number of transconjugants to the number of recipients.

3.1. Influence of temperature

To analyze the effect of cold temperature, the filters were inoculated with a mixture of 10^7 CFU mL^{-1} donor and 10^7 CFU mL^{-1} recipient and incubated overnight at temperatures ranging between 7 °C and 37 °C. At 7 °C no transconjugants were formed and at 10 °C only for one of the 4 filters one transconjugant was detected on the double selective plate (Figure 5.1a). The transconjugant status, being *L. monocytogenes* carrying the *tet*(M) gene was confirmed by PCR. Among the three remaining temperatures, the number of transconjugants ranged between $(2.75 \pm 0.50) \times 10^1$ CFU mL^{-1} and $(2.45 \pm 0.51) \times 10^3$ CFU mL^{-1} with the highest number of transconjugants at 22 °C. At 22 °C the highest number of recipients $((8.52 \pm 6.99) \times 10^8$ CFU mL$^{-1})$ was found. The number of donors ranged between $(3.13 \pm 1.41) \times 10^7$ CFU mL^{-1} and $(3.53 \pm 1.03) \times 10^8$ CFU mL^{-1}. The transfer ratio decreased with decreasing temperature, with almost a 2 log reduction in transfer ratio from $(1.12 \pm 0.51) \times 10^{-5}$ to 1.41×10^{-7} between 37 °C and 10 °C (Figure 5.1b).

3.2. Influence of modified atmosphere packaging

The influence of MAP was explored by three different experiments. In each experiment the same filter mating technique as above was used, but they differed from each other by the medium on which the filter was applied and by the inoculation density.

In the first experiment (Agar_High density), a mixture of 10^7 CFU mL^{-1} donor and 10^8 CFU mL^{-1} recipient was brought onto the filters which were subsequently placed on agar plates. The plates were placed in trays and packaged under three different conditions, air, 50% CO_2/50% N_2 and 100% N_2. The trays were subsequently placed at 7 °C and after 5 and 10 days the filters were analyzed (Figure 5.2a). In contrast to the previous experiment (influence of temperature) in which no transconjugants were observed after an incubation of the filters for 20 h at 7 °C, transconjugants were detected in this experiment in which the filters were incubated for 5 and 10 days at 7 °C. The number of transconjugants ranged between $(3.70 \pm 1.59) \times 10^2$ CFU mL^{-1} and $(1.28 \pm 0.74) \times 10^3$ CFU mL^{-1} for the three conditions on day 5 and on day 10 the range was between $(1.04 \pm 0.33) \times 10^3$ CFU mL^{-1} and $(7.85 \pm 3.23) \times 10^3$ CFU mL^{-1}. No statistically significant differences were found between the different atmospheres on the two time points, nor between the two time points for the different atmospheres. The lowest number of recipients was found on the filters that were incubated under the 50% CO_2/50% N_2 atmosphere. Both on day 5 and on day 10, the number of recipients remained below the number of recipients which was added on the filter. The donor was able to grow and reached under every condition an order of magnitude of 10^9 CFU mL^{-1}. Although it seems that the transfer ratio is higher under modified atmosphere conditions (order of magnitude 10^{-4} - 10^{-5}) than under ambient air (order of magnitude 10^{-6}), this was not confirmed statistically (Figure 5.2b and Table 5.2).
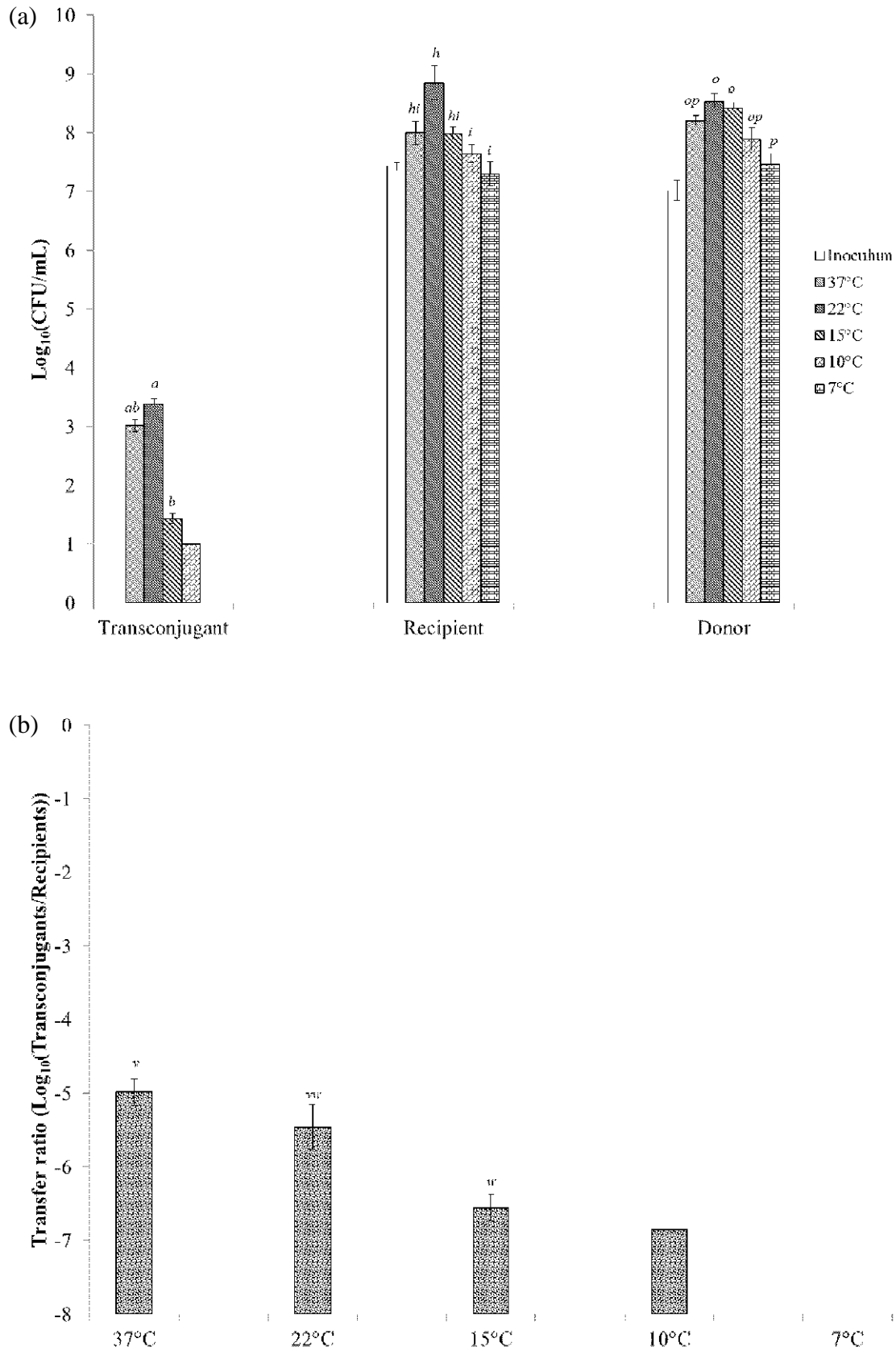
Figure 5.1. (a) Cell densities obtained on filters incubated on agar at different temperatures; (b) transfer ratio obtained on filters incubated on agar at different temperatures. Mean values sharing common lowercase letters are not significantly different (P <0.05).
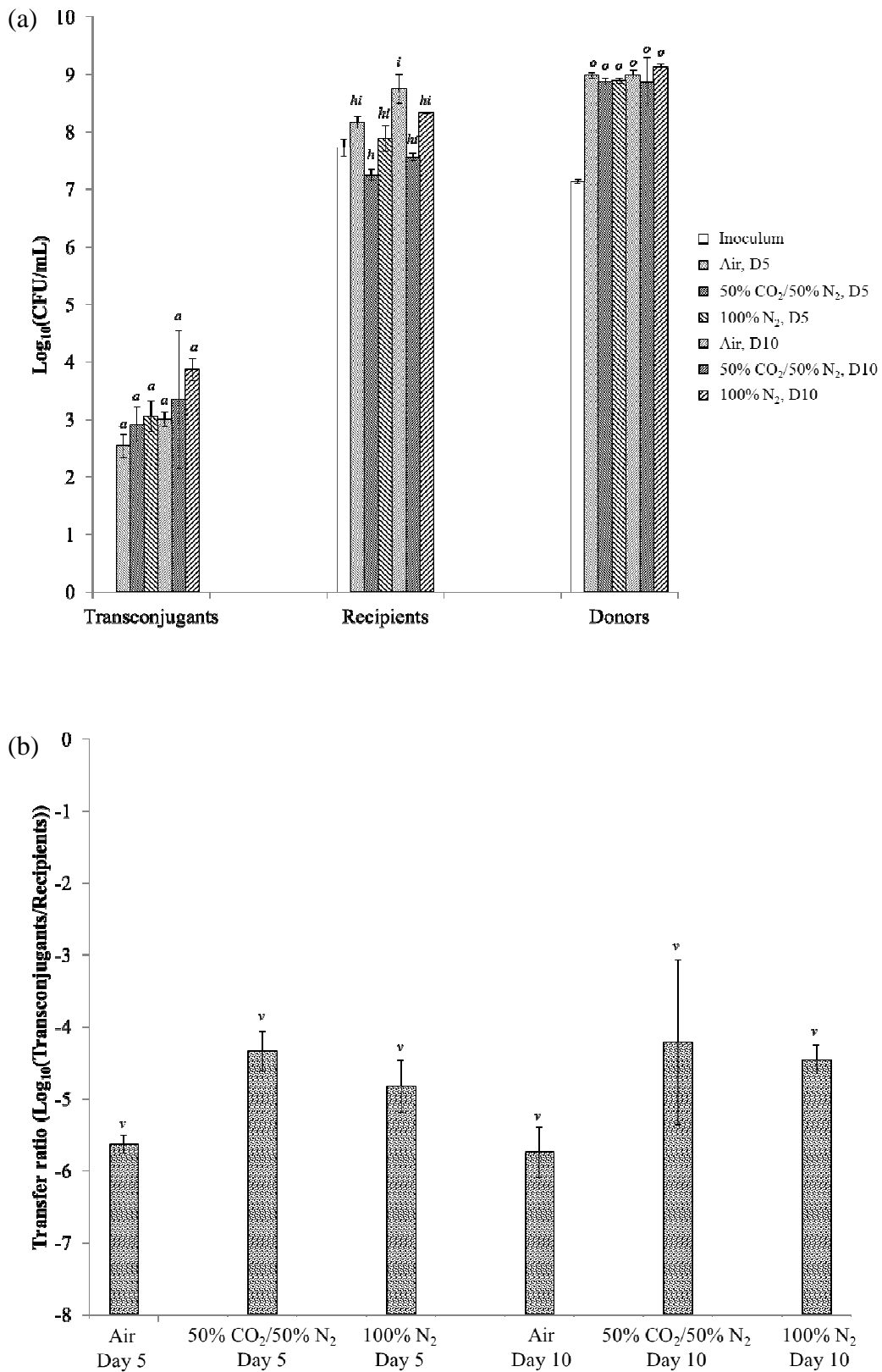
Figure 5.2. (a) Cell densities obtained on filters incubated on agar under different atmospheres at 7 °C for 5 and 10 days; (b) transfer ratio obtained on filters incubated for 5 and 10 days on agar under different atmospheres at 7 °C. Mean values sharing common lowercase letters are not significantly different (P <0.05).

In the second experiment (Ham_High density) the same high inoculation densities (10^7 CFU mL^{-1} donor and 10^8 CFU mL^{-1} recipient) were used and plasmid transfer was examined under the same conditions (air, 50% CO_2/50% N_2 and 100% N_2) after 5 days, but the filters were now placed on slices of cooked ham (Figure 5.3a). The number of transconjugants ranged from $(2.00 \pm 0.16) \times 10^2$ CFU mL^{-1} to $(7.55 \pm 4.45) \times 10^2$ CFU mL^{-1} with no statistically significant differences. For all three atmosphere conditions, the number of recipients decreased slightly compared to the inoculation density. The donor strain was able to grow under the three conditions as the number of donors detected on the filters had an order of magnitude of 10^8 CFU mL^{-1} with no statistically significant differences between the three conditions. The transfer ratio for the three conditions was comparable, with an order of magnitude of 10^{-5} (Fig 5.3b and table 5.2).

Figure 5.3. *(a) Cell densities obtained on filters inoculated with high cell densities and incubated on cooked ham under different atmospheres at 7 °C during 5 days; (b) Transfer ratio obtained on filters inoculated with high cell densities and incubated on cooked ham under different atmospheres at 7 °C during 5 days. Mean values sharing common lowercase letters are not significantly different (*P *<0.05).*

In the last experiment (Ham_Low density) the filters were inoculated with a low density mixture, containing on average $(5.26 \pm 0.56) \times 10^2$ CFU mL^{-1} donor and $(4.24 \pm 1.01) \times 10^2$ CFU mL^{-1} recipient, and placed on slices of cooked ham. Analysis was done after 5 and 10 days (Figure 5.4). Twice a transconjugant was detected on a filter which was incubated under 100% N_2 during 10 days. The transconjugant status of these two colonies was confirmed by PCR. After 5 days the number of recipients had not increased much compared to the inoculation density and the number of recipient for the 50% CO_2/50% N_2 condition had stayed below the inoculation level. However, no statistically significant differences were observed between the three conditions. After 10 days the number of recipients had increased to an order of magnitude of 10^3 - 10^4 CFU mL^{-1}, but again no statistically significant differences were observed between the three atmosphere conditions. The donor had already increased after 5 days (10^4 - 10^6 CFU mL^{-1}) and reached a density of 10^7 - 10^8 CFU mL^{-1} after 10 days. The two detected transconjugants yielded a transfer ratio of 10^{-5} (Table 5.2).
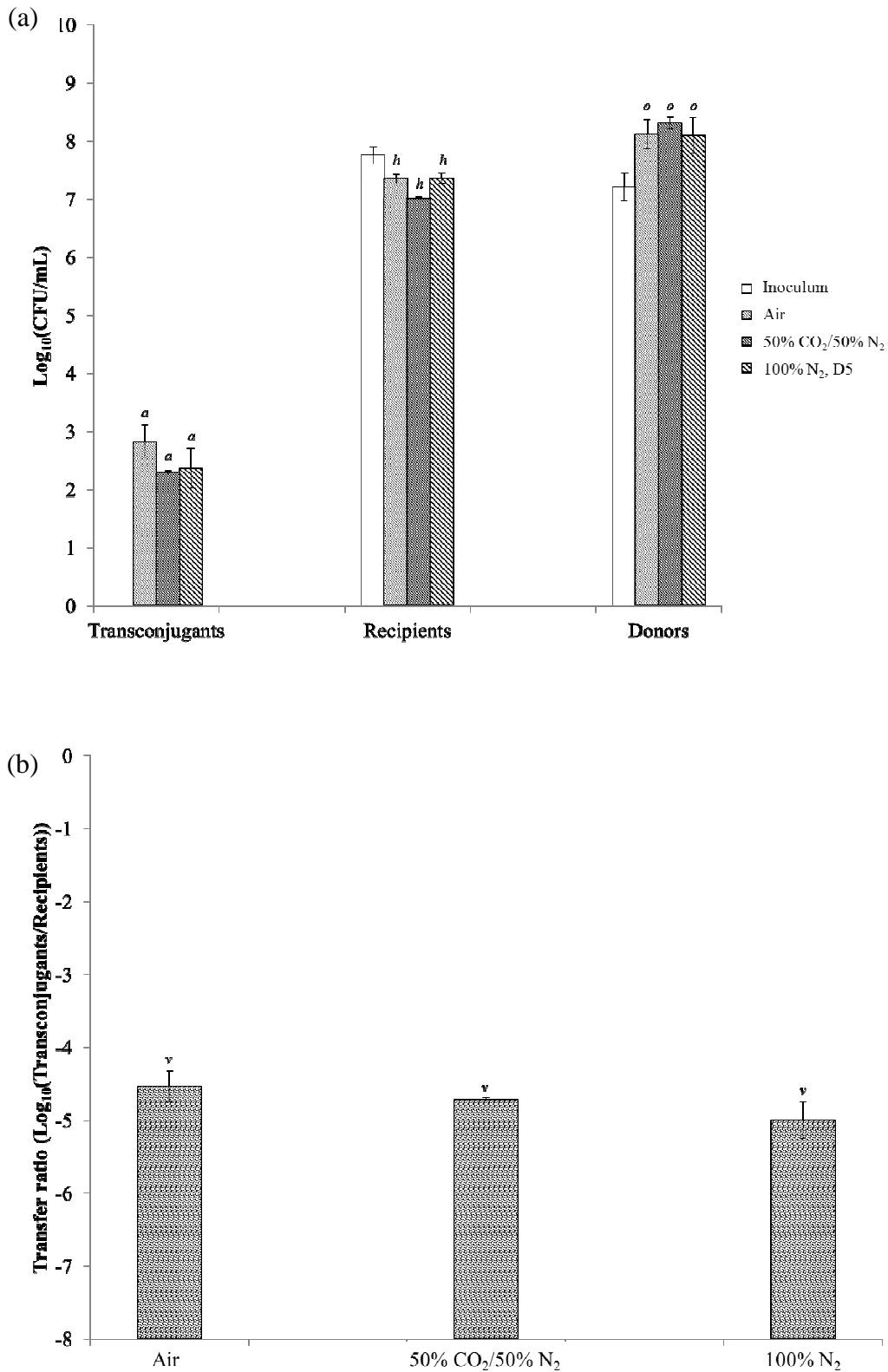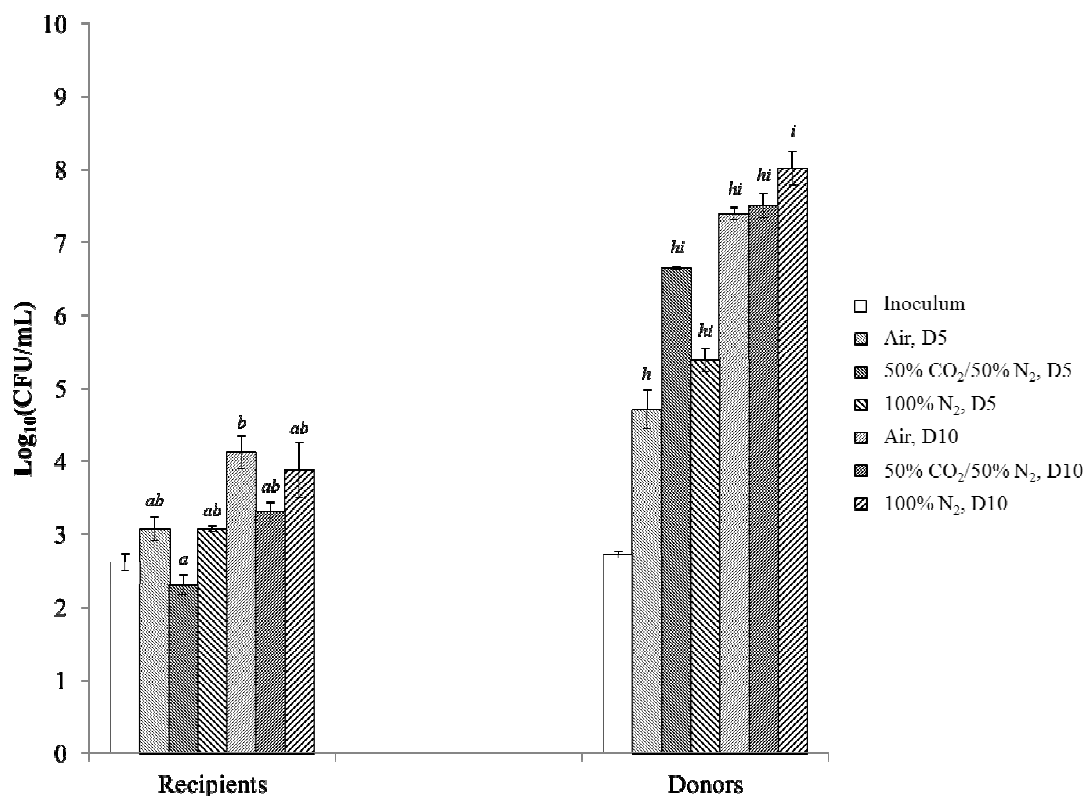

Figure 5.4. *Cell densities obtained on filters inoculated with low cell densities and incubated on cooked ham under different atmospheres at 7 °C during 5 and 10 days. Mean values sharing common lowercase letters are not significantly different (P <0.05).*

Table 5.2. Range of the transfer ratio (number of transconjugants/number of recipients) obtained in the different MAP experiments.

Experimental set-up	Incubation time (days)	Atmosphere	Transfer ratio range
Agar_High density	5	Air	$1.87 \times 10^{-6} - 3.29 \times 10^{-6}$
		50% CO_2/50% N_2	$2.93 \times 10^{-5} - 9.64 \times 10^{-5}$
		100% N_2	$5.74 \times 10^{-6} - 2.78 \times 10^{-5}$
	10	Air	$7.08 \times 10^{-7} - 3.34 \times 10^{-6}$
		50% CO_2/50% N_2	$2.94 \times 10^{-6} - 3.93 \times 10^{-4}$
		100% N_2	$2.08 \times 10^{-5} - 5.26 \times 10^{-5}$
Ham_High density	5	Air	$1.65 \times 10^{-5} - 4.32 \times 10^{-5}$
		50% CO_2/50% N_2	$1.80 \times 10^{-5} - 2.09 \times 10^{-5}$
		100% N_2	$5.94 \times 10^{-6} - 1.88 \times 10^{-5}$
Ham_Low density	5	Air	No transfer detected
		50% CO_2/50% N_2	No transfer detected
		100% N_2	No transfer detected
	10	Air	No transfer detected
		50% CO_2/50% N_2	No transfer detected
		100% N_2	$3.75 \times 10^{-5} - 4.14 \times 10^{-5}$

4. Discussion

The role of the food industry in the emergence of antibiotic resistance has recently been reviewed by Capita & Alonso-Calleja (2013) and Verraes *et al.* (2013). Although the use of antibiotics in the primary production is considered as a main risk factor, the influence of food processing may not be ignored. Sublethal food preservation stress has been shown to contribute to both the phenotypic antibiotic resistance as to the transfer of antibiotic resistance determinants (Mc Mahon *et al.*, 2007a; McMahon *et al.*, 2007b; Walsh *et al.*, 2008; Toomey *et al.*, 2009b; Al-Nabulsi *et al.*, 2011; Beuls *et al.*, 2012; Ganjian *et al.* 2012). In the case of antibiotic resistance transfer, conjugation is considered as the most important mechanism as conjugative or mobilizable plasmids are the most common transmission vectors for antibiotic resistance genes (Boerlin & Reid-Smith, 2008; Hawkey & Jones, 2009). In this study, the

effect of low temperature and of MAP on plasmid transfer was investigated. To our knowledge, the effect of MAP on plasmid transfer has never been evaluated before.

Antibiotic resistance transfer from *Lb. sakei* subsp. *sakei* to *L. monocytogenes* was studied in a temperature range of 7 °C – 37 °C. After 20 hours of incubation transfer was detected in the range of 10 °C – 37 °C. The results also indicate that transfer ratio decreases with decreasing temperature. This finding is in line with the general consensus that low temperatures have a negative effect on plasmid transfer (Fernandez-Astorga *et al.*, 1992). However, precaution is warranted when defining the range within which plasmid transfer occurs as this seems to be dependent of the experimental set-up. In this study transfer was not observed at 7 °C when the filters were incubated during 20 hours. In the subsequent experiment, however, transconjugants were detected after an incubation period of 5 and 10 days at 7 °C, yielding an average transfer ratio of 10^{-6} at both time points (Figure 5.2). Singleton & Anson (1981) also demonstrated that by prolonging the mating period, transfer could be detected at a lower temperature. Additionally, the nature of the transfer environment can play a role. The transfer ratios obtained in this study were about one log higher on cooked ham than on the agar plates (Figure 5.2b and Figure 5.3b). Walsh *et al.* (2008) observed that at 15 °C transfer only occurred in meat and not in milk or broth. Cocconcelli *et al.* (2003) followed antibiotic resistance gene transfer in *Enterococcus faecalis* during cheese and sausage fermentation at 10 and 30 °C. In the cheese model, transconjugants were observed after a longer period of time at 10 °C than at 30 °C. In sausages, higher transfer rates were obtained than in cheese. Furthermore, similar transconjugant kinetics were found in sausages at both temperatures. Rizzotti et al (2009) found that tetracycline resistance transfer took place at 30 °C between one of three *E. faecalis* donor strains and *Listeria innocua* on fresh pork meat and dry fermented sausage slice samples whereas no transfer was observed at 10 °C. Because of the diversity in experimental set-up of the previous mentioned studies, it is however very difficult to compare our results and draw general conclusions, urging the need for more studies in this field.

Further in this study, the effect of modified atmosphere packaging on plasmid transfer was analyzed. This was first done *in vitro* via filter matings on agar plates with high density inocula and subsequently *in situ* on cooked ham slices with both high and low density inocula. Cooked ham was chosen as food product because it can represent processed RTE foods that can be considered as high risk foods with regard to *L. monocytogenes* due to the possibility of contamination during processing or further handling and which have a prolonged storage time under refrigeration. In Belgium, the prevalence of *L. monocytogenes* on cooked meat products

was 4.9% during 1997 - 1998 and 1.1% during 2005 - 2007 (Uyttendaele *et al.*, 1999; Uyttendaele *et al.*, 2009). On EU level a prevalence of 2.07% at the end of shelf life has been reported for packaged heat-treated meat products (EFSA, 2013). The origin of contamination of cooked ham is most likely post processing. This was indicated in the study of Uyttendaele *et al.* (1999), who found a prevalence of 1.40% on cooked ham before slicing and 6.14% after slicing. In a recent outbreak of *L. monocytogenes* associated with cooked ham in Switzerland, the source of contamination was not the production plant, but a company where the slicing and the packaging was done (Hächler *et al.*, 2013). *Lactobacillus* spp. (predominantly *Lb. sakei* and *Lb. curvatus*) is one of the most important members of the spoilage microbiota of vacuum or modified atmosphere packaged cooked meats (Vermeiren *et al.*, 2004). *Lb. sakei* has for example been associated with spoilage of sliced cooked ham (Samelis *et al.*, 1998). This species is considered as one of the most psychrophilic species of lactobacilli as some strains are able to grow at 2–4 °C (Champomier-Vergès *et al.*, 2002). In the *in vitro* situation (agar – high density), *L. monocytogenes* was not able to grow under the 50% CO_2/50% N_2 modified atmosphere, not after 5 days nor after 10 days, while the donor strain grew under every condition to a density of 10^9 CFU mL^{-1}. Plasmid transfer was observed under every condition. Although it seemed that the transfer ratio is higher under modified atmosphere conditions (order of magnitude 10^{-4} - 10^{-5}) than under ambient air (order of magnitude 10^{-6}), this was not confirmed statistically (Figure 5.2b). Nevertheless, the results suggest that it is rather the absence of oxygen causing an effect on plasmid transfer than the presence of CO_2. From these results, it is clear that plasmid transfer can occur at least as easily under modified atmospheres as under air conditions. When filters with high density inocula were applied on the cooked ham (cooked ham – high density), *L. monocytogenes* was not able to grow after 5 days under any of the three conditions, while *Lb. sakei* subsp. *sakei* was again able to grow under every circumstance (Figure 5.3a). Under the three conditions, plasmid transfer had occurred and the obtained average transfer ratio had an order of magnitude of 10^{-5} (Figure 5.3b). Although, lower donor and recipient densities were obtained on the filters incubated on cooked ham, the transfer ratio was in the range that was reached in the *in vitro* experiment. These results indicate that cooked ham can represent an environment suitable for antibiotic resistance transfer between lactic acid bacteria and the pathogen *L. monocytogenes*.

All the experiments so far were performed with high density inocula to provide a proof of concept. These high densities of *L. monocytogenes* were far above the EU legal safety criteria which state that *L. monocytogenes* in RTE foods, other than those intended for infants and for

special medical purposes should not exceed the limit of 100 CFU g^{-1} throughout the shelf life (OJEU, 2005b). With regard to psychrotrophic lactic acid bacteria, there is a target value of 10^7 CFU g^{-1} at the end of shelf life. The food product should however only be rejected on condition that there are unacceptable sensory abnormalities (Uyttendaele *et al.*, 2010). Taking these values into account, an experiment with low densities was conducted to simulate a more realistic condition (Figure 5.4). After 5 days, the number of *L. monocytogenes* had slightly increased, except under the 50% CO_2/50% N_2 condition. After 10 days, *L. monocytogenes* obtained, under the aerobic and 100% N_2 conditions, a density in the order of magnitude of 10^4 CFU mL^{-1}. After 5 days, the number of donor bacteria had increased with at least 2 log and an increase with 5 log-6 log was obtained after 10 days. After 10 days and only under the 100% N_2 condition, a few transconjugants were detected. Under this condition, the highest bacterial density was observed on the filters. For successful plasmid transfer, there has to be cell-cell contact and the cells have to be metabolic active. There are no indications that the cells were not metabolic active as growth of both donor and recipient was observed. This suggests that close cell-cell contact, and thus bacterial density, was the determining factor. Although only two transconjugants were observed, this presented a transfer ratio in the order of magnitude of 10^{-5}, which is in the same range as the transfer ratios obtained in the above-mentioned MAP experiments with high density inocula. After 10 days however, the safety criteria (<100 CFU g^{-1}) for *L. monocytogenes* had been exceeded approximately a hundred times.

Overall, it can be concluded that antibiotic resistance can be transferred from *Lb. sakei* subsp. *sakei* to *L. monocytogenes* under low temperature and under MAP conditions. The results indicate the importance of respecting the cold chain as it seems that the risk of plasmid transfer increases with increasing temperature. Furthermore, the results suggested that density could be a determining factor. In this study, transfer was only observed under densities which exceeded the food safety criteria or guidelines, indicating that when these are respected with the aid of good manufacturing practice (GMP) and good hygiene practice (GHP), the chance of antibiotic resistance transfer under these circumstances is minimal, however not unimportant. In this respect, it is again important to respect the cold chain as on the one hand low temperatures reduce the growth of bacteria and on the other hand low temperatures are needed to guarantee the inhibiting effect of CO_2. Still, in order to conclude that the role of MAP in the contribution of the food production chain to the dissemination of antibiotic resistance determinants is indeed minimal more studies are necessary.

Chapter 6

General Discussion

Following headlines appeared in the media last year: "Superbacterie duikt meer en meer op in ons vlees" (http://www.deredactie.be, dd. 25/05/2013), "Antibiotic-Resistant 'Superbugs' Creep Into Nation's Food Supply" (http://www.cnbc.com, dd. 18/04/2013). The particular feature of superbugs is their multidrug resistance. Consequently, they are difficult or impossible to treat (Collignon, 2013). **But where does this antibiotic resistance come from?**

1. The food chain

In food production several critical points can be encountered which may contribute to the emergence and dissemination of antibiotic resistance. Main stages in the food production chain are primary production, processing, distribution and preparation.

1.1. Primary animal production, selection for antibiotic resistance and horizontal gene transfer

In the case of food from animal origin, the primary production includes the breeding and rearing of the animals intended for consumption. It is generally agreed that the use of antibiotics in the primary production is a main driving force in the emergence of antibiotic resistance, since at that stage there is already a selection for resistant bacteria.

The ways and the speed by which bacteria can become antibiotic resistant are both very intriguing, but also terrifying. Bacteria can acquire extra genetic material by three main mechanisms, conjugation, transduction and transformation. In the case of antibiotic resistance, conjugation plays a major role. In **Chapter 2**, we have demonstrated that a multiresistance plasmid with an environmental origin can be transferred successfully to *Salmonella* spp. and *Escherichia coli* O157:H7. A remarkable observation was that *Salmonella enterica* subsp. *enterica* serovar Enteritidis, which is considered to be a "susceptible" serovar, seemed to acquire the plasmid the most readily. *Salmonella enterica* subsp. *enterica* serovar Typhimurium, on the other hand, is often associated with multiresistance. The classic example is *S. enterica* serovar Typhimurium DT104 which carries five antibiotic resistance genes associated with the presence of the *Salmonella* genomic island 1. These resistance genes are located in a complex class 1 integron. Integrons are an example of the genius systems that bacteria possess to capture antibiotic resistance genes. In **Chapter 3**, a Belgian collection of STEC strains was screened for the presence of integrons and subsequently the present gene cassettes were identified. Although only two types of gene cassettes, encoding

resistance to streptomycin/spectinomycin and to trimethoprim, were retrieved in this specific collection, it is appropriate to approach integrons with caution as they are often associated with multiresistance and are found widespread in Gram-negative pathogens. Even though in our study integrons were only found among the human samples, there are multiple reports in the literature about integron-positive *E. coli* originating from food animals (swine, cattle, poultry, sheep, goat) (*e.g.* Zhao *et al.*, 2001; White *et al.*, 2002; Guerra *et al.*, 2003; Sáenz *et al.*, 2004; Sunde, 2005; Box *et al.*, 2005; Lapierre *et al.*, 2008; Povilonis *et al.*, 2010; Soufi *et al.*, 2011; Glenn *et al.*, 2012; Ben Sallem *et al.*, 2012; Marchant *et al.*, 2013; Ramos *et al.*, 2013) and food products, such as ready-to-eat salads in Portugal (Campos *et al.*, 2013), retail chicken products in Portugal (Silva *et al.*, 2012), raw chicken meat in Thailand (Chaisatit *et al.*, 2012), fish & seafood in Korea (Ryu *et al.*, 2012), retail meat products in China (Li *et al.*, 2011), traditional Egyptian cheese (Hammad *et al.*, 2009), turkey meat products in the USA (Khaitsa *et al.*, 2008). These studies involved both pathogenic as non-pathogenic *E. coli*.

Once antibiotic resistance has emerged in food animals it can reach humans by several routes, of which food is the most important one, but direct contact with the animals and the environment can also play a role. Several control measures can be applied to lower the development and dissemination of resistant bacteria among food animals and in food products. These are based on three fundamental aspects (Aarestrup *et al.*, 2008):

I)   Knowledge of the magnitude & nature of the problem. This knowledge can be gathered by monitoring antibiotic resistance as well as antibiotic usage. Recently, a Center of Expertise on Antimicrobial Consumption and Resistance in Animals (AMCRA) was founded in Belgium with the aim of protecting both public and animal health and welfare, and accomplishing a sustainable policy of veterinary antimicrobial use in Belgium. An example of the activities of AMCRA are the guides which have been prepared for different sectors (pigs, poultry and cattle) on animal health on farms, well-considered use of antibacterial agents and formularies.

II)   Limiting the selective pressure by controlling antibiotic usage. This can be obtained by for example altering the prescription and application policy and behavior or by banning antibiotics, such as the EU wide ban of antibiotics as additives in animal nutrition for growth promoting purposes (EC regulation 1831/2003 (OJEU, 2003a)).

III)   Controlling spread of resistant bacteria. Two measures related to this aspect are improving hygiene and setting thresholds for certain types of resistant bacteria. One of the forerunners in controlling the emergence and spread of antibiotic resistant bacteria is Denmark, where several control measures have been implemented, such as among others the ban on the routine prophylactic use of antimicrobials in animals, the limitation of the profit veterinarians can generate from the direct sale of drugs, restriction on the use of antimicrobials of particular public health significance, the development of veterinary treatment guidelines, the implementation of preventive veterinary medicinal strategies (Wegener, 2006).

1.2. *Food processing*

1.2.1. Legislation, self-checking, GHP and HACCP

In 2002, the European Union issued regulation (EC) No 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, also known as the general food law (OJEC, 2002). This regulation was implemented in Belgian law by the Royal Decision of 14th November 2003 (KB-14/11/2003) regarding self-checking (autocontrole), compulsory notification and traceability in the food chain (Belgisch Staatsblad, 2003). This self-checking system should include good hygiene practices (GHP), Hazard Analysis - Critical Control Point (HACCP) principles, policies, traceability and recall requirements in the specific company setting. Good hygiene practices comprise the conditions and measures necessary to ensure the safety and suitability of food at all stages of the food chain (FAO/WHO, 2007), while HACCP can be defined as a methodology that identifies, evaluates, and controls hazards that are significant for food safety (Jacxsens *et al.*, 2009). It is mandatory for all business operators with activities in the food chain to introduce, implement and sustain a self-checking system with the exception of the primary sector. This last sector has to perform controls on the hygiene requirements and has to keep registers as mentioned in the Royal Decision of 14th November 2003 (Belgisch Staatsblad, 2003). Food hygiene is also regulated on European level by the hygiene package, which consists out of three regulations, one on the hygiene of foodstuffs ((EC) No 852/2004), one with specific hygiene rules for food of animal origin ((EC) No 853/2004) and the last one with specific rules for the organization of official controls on products of animal origin intended for human consumption ((EC) No 854/2004) (OJEU, 2004a, b, c). Microbiological criteria (both food safety criteria as well as process

hygiene criteria) for foodstuffs have been laid down in regulation (EC) No 2073/2005 (OJEU, 2005b). It goes without saying that these (hygiene) regulations also contribute to the restriction of the transfer of antibiotic resistant bacteria to humans through food.

1.2.2. <u>Biofilms, multiple aspects to reflect on in the food industry</u>

One of the major concerns of the food industry is the presence of biofilms in a food processing environment, which can form a persisting source of contamination during food production. Both spoilage and pathogenic bacterial species can be involved. As an example, biofilms in an ice cream plant contained following Gram-negative bacteria: *Proteus*, *Enterobacter*, *Citrobacter*, *Shigella*, *Escherichia*, *Edwardsiella*, *Aeromonas*, *Plesiomonas*, *Moraxella*, *Pseudomonas* and *Alcaligenes* spp. and following Gram-positive bacteria: *Staphylococcus*, *Bacillus*, *Listeria* spp. and lactic acid bacteria such as *Streptococcus*, *Leuconostoc* or *Pediococcus* spp. (Gunduz & Tuncel, 2006). Furthermore, biofilms are considered to be hotspots for horizontal gene transfer (Sørensen *et al.*, 2005). In **Chapter 4**, high transfer rates of a multiresistance plasmid in biofilm models which are representative for biofilms in the food industry were found. This indicates the importance to not only consider biofilms as a source of contamination in the food processing environment, but also as a source for the further dissemination of antibiotic resistance due to increased plasmid transfer. It is therefore of uttermost importance to eliminate biofilms in the food industry. However, this is easier said than done. The standard method to remove biofilms is cleaning and disinfection. Cleaning comprises the removal of food debris and other residues that may contain microorganisms or promote microbial growth, while disinfection aims at diminishing the surface population of viable cells left after cleaning and prevent microbial growth on surfaces before production restart (Simões *et al.*, 2010). Up to 90% or more of surface-associated microorganisms can be removed by the cleaning process, however it is not suited to kill them (Chmielewski & Frank, 2003). Two aspects concerning the use of disinfectants deserve consideration, namely the higher resistance to these compounds in biofilms and the possible link between biocide usage and antibiotic resistance. Biofilm cells are in general more resistant to disinfectants than planktonic cells. Potential mechanisms involved in this resistance are transport limitations, which seem to be related mainly to interactions between the biocide and biofilm components; phenotypic adaptations of biofilm cells as a result of adaptive responses to sublethal concentrations of disinfectants; phenotypic adaptations of cells in a biofilm environment resulting from the expression of specific genes in response to their direct microenvironmental conditions; horizontal gene transfer of biocide resistance

genes and mutations; protection due to the presence of multiple species in the biofilm (reviewed by Bridier *et al.*, 2011). As reported in the introduction, the use of biocides can lead to the emergence of antibiotic resistance. This has been demonstrated multiple times under laboratory conditions (reviewed by SCENIHR, 2009). There is however still some ambiguity about this link.

The aforementioned issues associated with biocides drive the need for new biofilm control strategies. Some anti-biofilm strategies that have recently been explored are amongst other enzyme-based detergents, bacteriophages, essential oils, bacteriocins, quorum sensing inhibitors, etc. (Simões *et al.*, 2010; Bridier *et al.*, 2011; Giaouris *et al.*, 2014). Enzymes could be helpful in the cleaning process by promoting the natural degradation of the biofilm matrix (Bridier *et al.*, 2011). There are however some drawbacks (Simões *et al.*, 2010). The specificity in the enzyme mode of action makes the use of formulations containing several different enzymes essential for a successful biofilm control strategy. Furthermore, enzymes are expensive compared to chemicals. Bacteriophages have been successful in controlling biofilms of *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Listeria monocytogenes* (Soni & Nannapaneni, 2010). Essential oils are active volatile compounds that are produced as secondary metabolites by many herbs and spices (Giaouris *et al.*, 2014). Giaouris *et al.* (2014) gives an overview of studies which have demonstrated the anti-biofilm action of several essential oils and their components. However, further research is needed as increased bacterial biofilm formation after subinhibitory exposure to essential oil compounds has also been demonstrated (Sandasi *et al.*, 2008). Bacteriocins are ribosomally synthesized antimicrobial peptides produced by one bacterium that are active against other bacteria, either in the same species (narrow-spectrum), or across genera (broad-spectrum) (Cotter *et al.*, 2005). The biofilm control potential of bacteriocins has been studied for example against *L. monocytogenes* (García-Almendárez *et al.*, 2008; Winkelströter *et al.*, 2011; Gómez *et al.*, 2012). Quorum sensing inhibitors which inhibit biofilm formation are for example brominated furanones and acyl homoserine lactones (AHL) analogs (Sintim *et al.*, 2010). The indications that bacteria could possibly develop resistance against quorum sensing inhibitors (Defoirdt *et al.*, 2010) demonstrate the need for further research concerning the possibilities of resistance development against these mechanisms by bacteria.

1.2.3. <u>Minimal food processing</u>

The food industry increasingly applies minimal processing techniques to satisfy the consumer demand for healthy food with superior organoleptic properties. These methods change the inherent fresh-like quality characteristics of the food as little as possible (minimally), but at the same time provide the food product with a sufficient shelf life (Ohlsson, 1994). However, there is a lack of knowledge about how these techniques contribute to the emergence and the dissemination of antibiotic resistance in our food.

In food preservation, combination technology is applied. This means that the microorganisms present on the food are subjected to a combination of different hurdles, namely preservation techniques at low intensities, with the aim to prevent growth and proliferation of the undesired microorganisms. More than 60 possible hurdles have been described of which temperature (high or low), water activity (a_w), acidity (pH), redox potential (Eh), preservatives (*e.g.* nitrite, sorbate, sulphite), and competitive microorganisms (*e.g.* lactic acid bacteria) are the most important (Leistner, 2000). The effect of some of these hurdles (mainly temperature and pH) on plasmid transfer has been explored. Modified atmosphere packaging is a hurdle that is increasingly applied in food preservation, but which has to our knowledge not been implicated in studies on plasmid transfer. In **Chapter 5**, plasmid transfer under low temperature and under modified atmosphere conditions was evaluated. Our *in vitro* results (agar plates with high inoculum densities) indicated that the lower limit at which plasmid transfer occurs can vary according to the storage period. Although the modified atmosphere could prevent or retard the growth of the recipient strain (*L. monocytogenes*), it could not prevent plasmid transfer and transfer rates obtained under different atmosphere conditions were not significantly different. In other words, there is a risk of plasmid transfer during food preservation. This was also observed in the *in situ* experiments with cooked ham at high inoculum densities. At European level microbiological criteria are established to ensure food safety (EC No 2073/2005 (OJEU, 2005b)). For ready-to-eat foods able to support the growth of *L. monocytogenes* (other than those intended for infants and for special medical purposes) the threshold of *L. monocytogenes* is set at 100 CFU g^{-1} at the end of the shelf life. Keeping this in mind, an experiment with cooked ham was performed with similar low inoculum densities of both recipient and donor strain. *Lactobacillus sakei* subsp. *sakei* was used as donor strain. Only under the condition (10 days at 100% N_2) which yielded an average of 10^8 CFU mL^{-1} donor and 10^4 CFU mL^{-1} recipient, a few transconjugants were detected. For lactic acid bacteria, there are no strict criteria formulated, however there are guidelines which

mention a threshold of 10^7 CFU g^{-1} at the end of shelf life (Uyttendaele, 2010). The successful transfer in the former experiment was accompanied by numbers of the recipient strain which exceeded the legal microbiological criteria approximately a hundred times, and by numbers of the donor strain which can be associated with food spoilage. These findings indicate that under normal circumstances the risk of plasmid transfer is minimal. Further research is needed to resolve if it is negligible as well.

1.2.4. SOS response

Stress encountered by bacteria during food processing and preservation may trigger bacterial responses leading to enhanced survival. An example of such a mechanism is the SOS response, a global regulatory network targeted at addressing DNA damage (Erill *et al.*, 2007). The SOS response from a food safety perspective has recently been reviewed by van der Veen & Abee (2011). Stress factors that possibly provoke the SOS response include food preservation factors (*e.g.* UV-radiation, preservatives), food processing factors (*e.g.* heat, high pressure) and/or cleaning agents such as oxidative compounds, which can result in increased stress resistance and induction of genetic diversity (van der Veen & Abee, 2011). Several aspects addressed in this thesis are associated in some way with the SOS response. Antibiotics can be inducers of the SOS response resulting in antibiotic resistance by the formation of persisters, enhancing the mutation rate or by stimulating horizontal gene transfer (reviewed by Rodríguez-Rojas *et al.*, 2013). Furthermore, it was recently discovered that the SOS response controls integron recombination (Guerin *et al.*, 2009). Both conjugation and transformation can induce the SOS response, hereby triggering integrase expression (Baharoglu *et al.*, 2010, 2012). The SOS response also plays a role in biofilm formation as has been demonstrated for example in *L. monocytogenes* (van der Veen & Abee, 2010) and in *P. aeruginosa* (Chellappa *et al.*, 2013). In heterogeneous and nutrient-deprived biofilm microenvironments, the induction of the SOS response can lead to biofilm-specific high tolerance to the antibiotic ofloxacin (Bernier *et al.*, 2013). The above clearly shows that the SOS response plays an important role in the adaptive capacity of bacteria during adverse conditions.

1.3. Antibiotic resistance transfer during food production and preservation: the example of cooked ham

Cooked ham is generally prepared from porcine whole muscles. The raw material has to pass several processing steps before cooked ham is obtained. The main processes are brining, tumbling, cooking, cooling, slicing and packaging. The bacterial count of the raw material should be as low as possible, preferably between $10^2 – 10^4$ CFU g^{-1}, as this helps to keep the bacterial count low throughout the manufacturing process and it greatly enhances the shelf life of the cooked product (Feiner, 2006). During brining, a solution of sodium chloride, nitrites and possibly other ingredients are injected into the meat. The brine injection level and the ingredients used are characteristic for each product and determine the cooked ham quality (Casiraghi *et al.*, 2007). In order to avoid bacterial growth in the injected meat, it is important that the brine is kept at a low temperature (Feiner, 2006). Tumbling is a mechanical operation by which the brine is evenly distributed in the meat and proteins are extracted from muscle fibres (Casiraghi *et al.*, 2007). This is also best done at low temperature (Feiner, 2006). Before cooking, the tumbled meat is formed by placing it in moulds or casings. Sometimes the tumbled meat is first vacuum packed before putting it into the moulds. The cooked product can yield in this way long shelf lives as no recontamination can occur after cooking under normal conditions. The cooking normally takes place at 74 - 80 °C until a core temperature of 69 - 72 °C is obtained and serves to denature proteins, stabilize the curing colour, intensify the flavour, improve the texture and destroy pathogens (Feiner, 2006). After cooking, it is essential to bring the meat quickly to a temperature below 10 °C as spores who have survived the cooking process can germinate and grow at temperatures above 10 °C. The products are usually first showered or bathed in cold water before placing them in a blast chiller. Slicing represents the stage with the highest risk of contamination. This risk can be decreased by providing a strict separation between pre- and post-cook areas, by maintaining high personal hygiene and by applying a positive or negative air pressure in the slicing rooms (Feiner, 2006). The formation of condensation has to be avoided as well. Sliced products are predominantly packed under modified atmospheres so that the individual slices do not stick together and the product is not squeezed as if packed under vacuum. For an optimal shelf life and to prevent bacterial growth as much as possible, packed products should be stored between -1 and 4 °C (Feiner, 2006).

Samelis *et al.* (1998) followed the microbiology at several stages during the manufacturing of cooked ham. At the beginning of the process pseudomonads dominated the microbiota, while

during tumbling, lactic acid bacteria became dominant. It was also during tumbling that cross-contamination of the meat with *L. monocytogenes* occurred. *L. monocytogenes* was eliminated by the heat processing step and was absent during storage. After heat processing and cooling only lactic acid bacteria were able to grow during storage in vacuum packs. The microbiota of Belgian artisan cooked ham packed under modified atmosphere consisted of *Leuconostoc* spp., *Carnobacterium* spp. and *Brochothrix thermosphacta* (Vasilopoulos *et al.*, 2010). These bacteria were also detected in raw tumbled meat suggesting the presence of a "house microbiota", consisting of microorganisms which are introduced onto surfaces and into the environment of the processing line through contact with the meat and of its handling by the personnel (Vasilopoulos *et al.*, 2010). As the heat treatment during the processing of cooked ham will kill most vegetative cells, it is more likely that recontamination of cooked meat products occurs during handling, slicing and/or packaging. Audenaert *et al.* (2010) reported a common lactic acid bacteria contamination in a study of cooked ham, turkey and chicken products. The processing occurred on different locations for poultry and pork, while the slicing and the packaging took place in the same production plant. Contamination with the pathogen *L. monocytogenes* also occurs most likely during post-processing (Uyttendaele *et al.*, 1999). The source of contamination in a recent *L. monocytogenes* outbreak in Switzerland associated with cooked ham, was not the production plant itself, but a company where the slicing and the packaging was done (Hächler *et al.*, 2013).

Based on the results obtained in **Chapter 5** the risk for antibiotic resistance transfer seems highest during storage under modified atmosphere as the bacteria reach the highest densities at this stage. Raw material can contain lactic acid bacteria in an order of magnitude of 3 log CFU g^{-1} (Samelis *et al.*, 1998; Vasilopoulos *et al.*, 2010). In the experiments conducted in **Chapter 5**, transfer on cooked ham was only detected when a bacterial density of 8 log CFU mL^{-1} was reached. In the study of Samelis *et al.* (1998) this density was reached after storage of vacuum packed, cooked ham during 6 to 12 days. For MAP artisan-type cooked ham packages, it took several weeks to obtain this density at 4 and 7 °C. However, during this period the practical threshold of 6 log CFU g^{-1} for total viable bacterial counts, as used in artisan-type ham practice for rejection of the product, was already exceeded (Vasilopoulos *et al.*, 2008). The lowest density of the recipient bacterium at which transfer was observed in **Chapter 5** was in the order of magnitude of 4 log CFU mL^{-1}. In a recent European survey on the prevalence of *L. monocytogenes* in certain ready-to-eat foods, densities of > 4 log CFU g^{-1} were observed in 0.06% of the analyzed packaged heat-treated meat product samples (EFSA, 2013). In **Chapter 5** the tip of the veil was lifted by

demonstrating that antibiotic resistance transfer can take place on food products packed under modified atmosphere. Further research is necessary to assess the importance of this phenomenon.

1.4. Consumer

Can we still enjoy our food as "bon vivants"?

Of course, as long as you are aware of the presence of bacteria and you act accordingly to minimize the risk of acquiring a foodborne disease.

In the fight against foodborne bacterial infections and intoxications, a part of the responsibility lies, obviously, with the consumer. In the time period 2007-2011, the setting "household/domestic kitchen" was reported to be involved in 32.7 – 38.7% of the foodborne outbreaks (EFSA/ECDC, 2009b, 2010, 2011, 2012, 2013). There are several factors contributing to these high frequencies, *e.g.* the majority of the food we eat is prepared at home, a false sense of being safe from foodborne illnesses with insufficient attention to general hygiene principles as a consequence, the multifunctionality of the kitchen, too high refrigerator temperatures (Byrd-Bredbenner *et al.*, 2013). Antibiotic resistance is not often integrated in the bacteriological screening of domestic kitchens. Marshall *et al.* (2012) found in their screening of kitchen sites overall no significant differences or trends in antibiotic resistance between users and non-users of biocide agents. In another study, *Cronobacter sakazakii* was found to be present in 26.9% of the evaluated domestic kitchens and overall a resistance to two or more antibiotics was observed (Kilonzo-Nthenge *et al.*, 2012). The same author found in a previous screening of domestic refrigerators no *L. monocytogenes*, while several species belonging to the *Enterobacteriaceae* were detected, most of which are not usually associated with foodborne pathogens and are considered non-pathogenic to healthy adults, except for *Enterobacter sakazakii* (2.2%) and *Yersinia enterocolitica* (0.7%) (Kilonzo-Nthenge *et al.*, 2008). Among the *Enterobacteriaceae* isolates antibiotic resistance was observed, with multidrug resistance found only in *Klebsiella* spp. The occurrence of antibiotic resistance gene transfer in a domestic kitchen environment has been investigated very seldom. Kruse & Sørum (1994) demonstrated that plasmid transfer could take place on a hand towel and that cutting boards can transfer recipient strains to food products on which subsequently successful transfer can take place. Transfer was also detected in the remnants on the cutting board.

To prevent foodborne diseases at consumer level, five keys have been published by the World Health Organization (WHO): keep clean, separate raw and cooked food, cook food thoroughly, keep food at safe temperatures, use safe water and raw materials (http://www.who.int/foodsafety/consumer/5keys/en/). It goes without saying that these measures will also constrain the transfer of antibiotic resistance.

2. Does the story end with antibiotic resistant pathogens present in our food?

No, absolutely not.

Although our main concern are the pathogenic antibiotic resistant bacteria as they represent the main direct threat to the public health, the role of the commensal bacteria present in our food should not be underestimated. Commensals are bacteria which belong physiologically to the human or animal microbiota and which are not primarily considered as pathogenic for their host. The most studied commensal species are *E. coli* and *Enterococcus* spp. Both species have a number of characteristics in common: I) they can be found in the gastrointestinal system of humans and animals; II) they are possible food contaminants; III) they may carry transmissible resistance genes; IV) they are facultative pathogens. Commensals can pose an indirect hazard if they carry transferable antibiotic resistance genes, which they can pass to human pathogenic bacteria. According to some, the commensal antibiotic resistance reservoir can be considered a more global threat to health than the direct selection pressure on the pathogens themselves, as the occasional *de novo* development of resistance in a pathogen may be less frequent and less impactful than the constant gene traffic from the vast commensal reservoir into the relatively small pathogen pool (Boerlin & Reid-Smith, 2008). Furthermore, it seems that multidrug resistant commensal *E. coli* has the highest significance in the food animal industry, where it may act as reservoir for intra- and interspecies exchange and as a source for dissemination of multidrug resistant determinants through contaminated food to humans (Szmolka & Nagy, 2013). Werner *et al.* (2013) has recently reviewed the role of antibiotic resistant enterococci as "resistance gene trafficker" highlighting the importance of preventing the development of new resistant strains and the transfer of multiple resistant enterococci via the food chain.

A suitable place where bacteria can transfer their antibiotic resistance genes is the human gastrointestinal tract. This has been demonstrated by several model systems, such as a

single-stage continuous fermenter system to simulate the microbial ecosystem of the proximal infant colon (Haug *et al.*, 2011), an *in situ* continuous flow culture system, simulating the human caecum and the ascending colon (Smet *et al.*, 2011), but also different *in vivo* models have been applied (Schjørring & Krogfelt, 2011). It is generally accepted that the human gut is likewise a reservoir of antibiotic resistance genes and that there is an interplay among environmental, food, and gut microbiota of humans and animals whereby genetic exchanges can occur at any step (Figure 6.1).
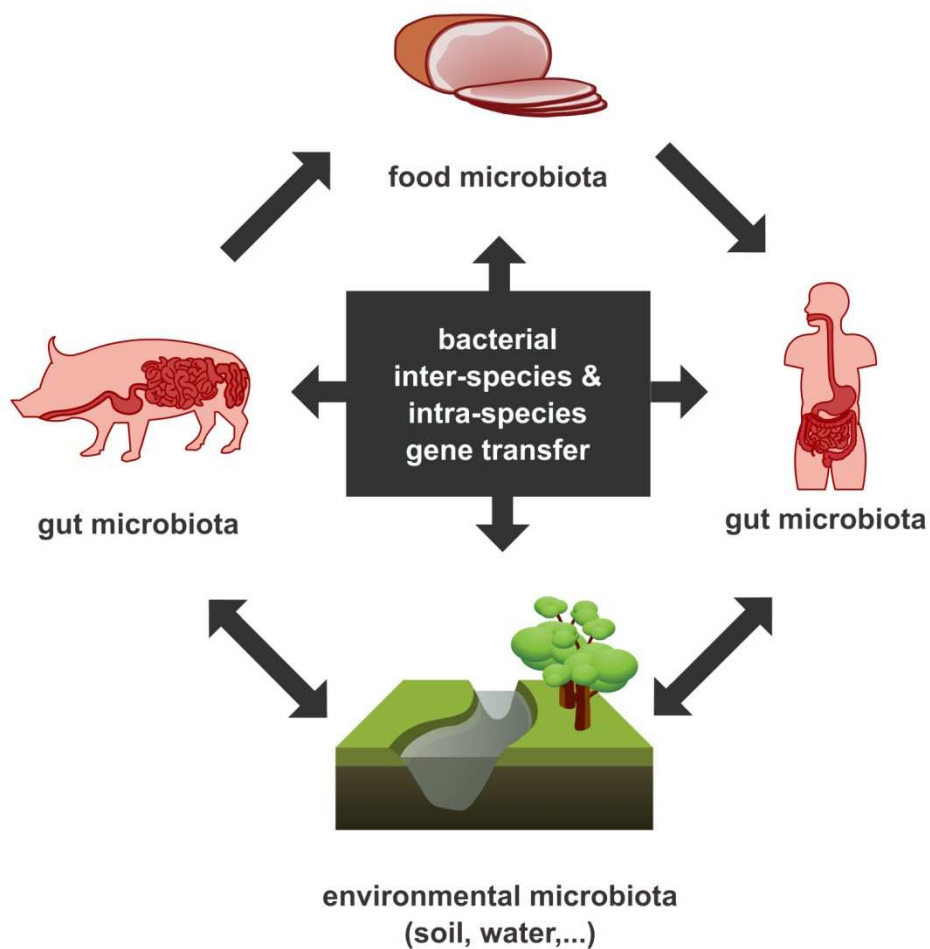

Figure 6.1. *Graphic representation of the interplay among environmental, food, and gut microbiota of humans and animals. Genetic exchanges can occur at any step. (Adapted from Devirgiliis* et al.*, 2011)*

3. Applied methodology

In this doctoral work two techniques were applied for the quantification of plasmid transfer: plating and flow cytometry. Plating, the traditional detection technique in bacteriology, is time consuming and unable to detect VBNC bacteria. The use of flow cytometry to study conjugation is a relatively new technique which was first described in 2003 (Sørensen *et al.*, 2003). Major advantages of this technique are its rapidity and the possibility to detect VBNC bacteria. However, there are also some disadvantages. The setting of the gates is performed arbitrarily, which can complicate the comparison of results. Another drawback is, that in order to be able to analyze conjugation by flow cytometry, a reporter system has to be integrated. In this doctoral work, this meant manipulating the donor strain and the plasmid. Concerning the insertion of the mini-Tn*5*-Km-P$_{A1-04/03}$*::gfp* cassette, it is not known where it has integrated in the plasmid. However, with the current sequencing techniques it is nowadays possible to sequence the plasmid to detect where it has inserted and if this could influence its transferability or stability.

In Chapter 2 and 4 where flow cytometry was used to quantify plasmid transfer, the transfer ratio was expressed as the ratio of the number of transconjugants to the total cell number. In theory, it would be possible to induce *gfp* expression in the donor bacteria by the addition of isopropyl-thio-β-D-galactoside (IPTG). In pseudomonads however, it has previously been shown that *lac*-type promoters are less efficiently induced by IPTG (Sørensen *et al.*, 2003).

Another factor to keep in mind is that the fluorescence of GFP can be impaired by some environmental conditions, such as high salt, low pH and lack of oxygen (Sørensen *et al.*, 2005). Considering the case of low oxygen, Hansen *et al.* (2001) have demonstrated that when shifting an anaerobically grown (non-fluorescent) >50 µm thick *Streptococcus gordonii* biofilm to aerobic conditions, GFP fluorescence could be detected within 4 minutes, reaching a maximum over the next 16 minutes. In Chapter 2, cells were removed from the filters by vortexing and in Chapter 4 biofilms were removed from the attachment material and mechanically disrupted. These manipulations were conducted under aerobic conditions giving the cells the opportunity to become fluorescent. Recently, a new fluorescent reporter system allowing quantitative analysis of plasmid transfer under both aerobic and anaerobic conditions has been designed (Król *et al.*, 2010).

In an ideal situation, anyone planning conjugation experiments should consider these aspects carefully for the experimental design. However, practical considerations cannot be neglected.

The biomass acquired in filter mating experiments (Chapter 2 and Chapter 5) can also be considered as a biofilm. The difference between filter mating and the biofilm models used in Chapter 4 is that the biofilm on the filter is formed under static conditions while the biofilms in Chapter 4 were formed under flow condition. The preference for one of the approaches depends on the research question.

In this doctoral work, filter matings were applied to study plasmid transfer on a food product. This was done to obtain a standardized methodology to apply and recover the bacteria, but also to give the bacteria the best chance of making contact. Other methods have been described in literature. Walsh *et al.* (2008), for example, verified that the ground meat they used were *Salmonella* free and *E. coli* free. Ground meat samples were inoculated by immersing them in an inoculating suspension after which they were drained and reminced. Gazzola *et al.* (2012), who used a fermented sausage model to assess horizontal gene transfer, sterilized pork meat batter by gamma ray irradiation treatment (6 KGy) to eliminate the adventitious microbiota present in the raw meat. Bertsch *et al.* (2013) spread the inoculation suspension on the surface of smoked salmon and of smear-ripened cheese. After incubation, the cheese smear was scraped off with a sterile knife whereas the complete salmon sample was analyzed. By applying the filter mating method, we gave the bacteria the best chance to make contact, however, the methods applied by Walsh *et al.* (2008) and by Bertsch *et al.* (2013) approach the real life situation more closely.

4. Conclusion

The topic of this PhD research was the study of antibiotic resistance transfer during food production and preservation, addressing a few aspects of the dissemination of antibiotic resistance from farm to fork. Several nice findings were obtained. First of all, the transfer of an environmental multiresistance plasmid (originally isolated from a wastewater treatment plant) to foodborne pathogens has shown that the environment and the food production chain are not strictly separated niches, but that they certainly can interact with each other. Secondly, the diversity of the gene cassettes present in integrons in a Belgian collection of STEC seemed to be limited. As such, this might be positive, however, more than 90% of the integron-positive STEC strains displayed resistance to three or more antibiotics. This clearly illustrates the importance of monitoring antibiotic resistance in STEC, a research area which has not received a lot of attention. Thirdly, the biofilm research reveals that existing biofilms which are representative for biofilms in the food industry can serve as a source or a receiver for multiresistance plasmids. This is without a doubt a point requiring further attention. Finally, modified atmosphere packaging did not seem to be a parameter preventing plasmid transfer. Plasmid transfer even took place on cooked ham packed under modified atmosphere. It has to be noted, however, that plasmid transfer was observed only with densities that greatly exceeded food safety criteria/guidelines.

Considering the farm to fork route, it is clear that the use of antibiotics in the primary production has an immense impact on the emergence of antibiotic resistance. This is certainly a point that requires further attention in the fight against antibiotic resistance. It is however imperative to always keep in mind that the different stages in the farm to fork concept are not strictly defined units, but that interactions may occur. Once antibiotic resistance has emerged, it is important to look at the factors that can contribute to a further spread of antibiotic resistance. From this PhD research, it can be concluded that during food production and preservation there are definitely factors contributing to a further dissemination of antibiotic resistance by means of plasmid transfer. These conclusions, however, only relate to the model systems applied in this PhD research, indicating the need for further research on this topic. It would be interesting to apply the biofilm reactor in other experimental approaches in which more realistic situations are mimicked by adapting for example the temperature and/or the medium. Other model organisms should also be tested. To our knowledge, it is the first time that the effect of MAP conditions on plasmid transfer has been explored. Again, more

research with other food products and other model organisms is necessary to confirm our findings on the role of MAP in the dissemination of antibiotic resistance determinants.

Abstract

Antibiotic resistance is a worldwide public health problem. The dissemination of antibiotic resistance results from an interplay of factors between humans, animals, food and environment. There are strong indications that the use of antibiotics in primary production contributes to human infections with antibiotic resistant bacteria. Food serves hereby as an important vector. Regarding the dissemination of antibiotic resistance through food, a distinction exists between the direct risk, which refers to the dissemination of antibiotic resistant bacteria themselves, including the foodborne pathogens, and the indirect risk, which comprises the dissemination of antibiotic resistance genes. Although there are three main mechanisms by which bacteria can obtain exogenous genes, namely conjugation, transformation and transduction, it is assumed that conjugation is the most important mechanism concerning antibiotic resistance transfer. Conjugation involves the transfer of genetic elements from a donor to a recipient. The genetic element most frequently transferred via conjugation is a plasmid. The contribution of food production and preservation to antibiotic resistance transfer by means of plasmids, has however only been scarcely studied. This topic represents the focus of this doctoral work.

In a first phase, the transfer of a multiresistance plasmid to foodborne pathogens was analyzed (Chapter 2). The plasmid, pB10, originally isolated from a wastewater treatment plant, contains resistance genes against the antibiotics streptomycin, amoxicillin, tetracycline and sulfonamides. A *Pseudomonas putida* strain was used as donor. *P. putida* is a typical inhabitant of water and soil, but can also be associated with food spoilage. A selection of *Salmonella* and *Escherichia coli* O157:H7 strains, both notorious foodborne pathogens, was chosen as recipient. Conjugation was analyzed by plating and by flow cytometry. For 14 of the 15 analyzed strains transconjugants were detected. The transfer ratio, *i.e.* the ratio of the number of transconjugants to the total cell count, seemed to be recipient strain dependent and could reach an order of magnitude of 10^{-2}, in other words one out of 100 bacteria obtained the plasmid. Based on the antibiotic susceptibility profiles of the recipients and the transconjugants it could be confirmed that the bacteria, after obtaining the plasmid, became resistant against abovementioned antibiotics.

Integrons are genetic elements that are often associated with plasmids. They are a fine example of the fascinating way by which bacteria can acquire and further disseminate antibiotic resistance. In Chapter 3 a Belgian collection of Shiga-toxin producing *E. coli* (STEC) was screened for the presence of integrons. STEC is considered to be the most

important group of emerging foodborne pathogens. Antibiotic resistance in STEC, however, is hardly investigated. Integrons were detected in 7.5% of the analyzed strains, all from human origin. They were all class 1 integrons, which is the most common class in Gram-negative bacteria. Further characterization demonstrated that the detected integrons carried antibiotic resistance genes against two types of antibiotics, namely streptomycin/spectinomycin and trimethoprim. This would be good news, but analysis of the antibiotic susceptibility profiles showed that 91.3% of the integron-positive strains showed resistance to at least three different antibiotics. On the other hand, 77.0% of the integron-negative strains were susceptible for all the tested antibiotics.

In the food industry biofilms can form a persistent source of contamination, which may contribute to food spoilage, damage the equipment and which may constitute a risk to human health if pathogenic bacteria are involved. Moreover, these structures are considered as hotspots for plasmid transfer, whereby they can contribute to the dissemination of antibiotic resistance. In Chapter 4, the transfer of the multiresistance plasmid, pB10, was examined in biofilm models, representative of biofilms in the food industry. Two different flow configurations (flow-through and drip-flow) and three attachment materials (silicone, glass and stainless steel) were used. Just as in Chapter 2, *P. putida* was used as donor and *E. coli* as recipient. The inoculation strategy comprised first the formation of a biofilm with one of the two bacterial species and subsequently the application of the second bacterial species. This way, plasmid transfer was studied by means, on the one hand, of a biofilm with plasmid donating capacity and, on the other hand, of a biofilm with plasmid receiving capacity. High transfer ratios (the ratio of the number of transconjugants to the total cell number) were obtained, which could reach an order of magnitude of 10^{-1}.

To provide food with a sufficient shelf life, preservation techniques are used that prevent the outgrowth of bacteria present on food. Two such techniques are low temperature and modified atmosphere packaging (MAP). In Chapter 5, the effect of these two techniques on plasmid transfer was studied in a Gram-positive model. *Lactobacillus sakei* subsp. *sakei*, a typical Gram-positive spoilage organism, was used as donor and *Listeria monocytogenes*, a Gram-positive psychrotrophic pathogen, was used as recipient. Both species can be found on ready-to-eat foods, packaged under modified atmosphere. Concerning temperature, plasmid transfer was observed in a range between 10 °C and 37 °C. However, the lower limit could be decreased by extending the incubation period. To examine the effect of modified atmosphere

three gas compositions (air, 50% CO_2/50% N_2 and 100% N_2) were applied. When high inoculum densities were used, plasmid transfer was observed under each condition, both *in vitro*, on agar plates, as *in situ*, on slices of cooked ham. To simulate a more realistic situation, plasmid transfer was also analyzed on cooked ham with low inoculum densities. Transfer was observed only under the 100% N_2 condition after ten days incubation. Under this condition, the highest bacterial density was obtained. In the MAP experiments the transfer ratio, expressed as the ratio of the number of transconjugants to the number of recipients, was of the order of magnitude of 10^{-4} - 10^{-6}. It should be noted though that transfer was only observed with donor and recipient densities which exceed the food safety criteria or guidelines. If these criteria/guidelines can be guaranteed, the contribution to antibiotic resistance dissemination seems to be minimal.

This PhD research highlighted a small aspect of the factors involved in the problem of antibiotic resistance dissemination. Nevertheless, there were a number of important findings. First of all, it was shown that the environment and the food are not strictly defined niches, but that they certainly can interact with each other. Secondly, it appeared important to further monitor integrons, as these are often associated with mobile genetic elements that can carry additional antibiotic resistance genes. Furthermore, it became clear that biofilms are not only a source of contamination in the food industry, but the risk of antibiotic resistance dissemination by plasmid transfer in biofilms should also be acknowledged. Finally, two commonly used preservation techniques which prevent bacterial growth in the food industry, do not necessarily seem to prevent plasmid transfer.

Further research with other model systems is, however, necessary to expand our knowledge on the role that food production and preservation play in the dissemination of antibiotic resistance.

Samenvatting

Antibioticumresistentie is een wereldwijd probleem voor de volksgezondheid. De verspreiding van antibioticumresistentie resulteert uit een samenspel van factoren tussen mens, dier, voeding en omgeving. Er zijn sterke aanwijzingen dat het gebruik van antibiotica in de primaire productie bijdraagt aan humane infecties met antibioticumresistente bacteriën, waarbij voeding een belangrijke overdrager is. Wat de verspreiding van antibioticumresistentie via de voeding betreft, is er een onderscheid tussen het directe risico, dat betrekking heeft op de verspreiding van antibioticumresistente bacteriën zelf, waaronder de voedselgebonden pathogenen, en het indirecte risico, waarmee gedoeld wordt op de verspreiding van antibioticumresistentiegenen. Hoewel er drie belangrijke mechanismen bestaan waardoor bacteriën exogene genen kunnen verkrijgen, zijnde conjugatie, transformatie en transductie, wordt er aangenomen dat conjugatie in het geval van de overdracht van antibioticumresistentie het belangrijkste mechanisme is. Tijdens conjugatie worden er genetische elementen overgedragen van een donor naar een acceptor. Het genetisch element dat het vaakst via conjugatie wordt overgedragen is een plasmide. Naar de bijdrage die de voedselproductie en -bewaring levert aan de overdracht van antibioticumresistentie d.m.v. plasmiden, is er echter weinig onderzoek uitgevoerd. Hierin ligt dan ook de focus van dit doctoraatswerk.

In een eerste fase werd de overdracht van een multiresistent plasmide naar voedselpathogenen geanalyseerd (Hoofdstuk 2). Het plasmide, pB10, oorspronkelijk geïsoleerd uit een afvalwaterzuiveringsinstallatie, bevat resistentiegenen tegen de antibiotica streptomycine, amoxicilline, tetracycline en sulfonamides. Als donor werd er gebruikt gemaakt van een *Pseudomonas putida* stam. *P. putida* is een typische bewoner van water en bodem, maar kan ook betrokken zijn bij voedselbederf. Als acceptor werd er gekozen voor een selectie van *Salmonella* en *Escherichia coli* O157:H7 stammen, beide beruchte voedselpathogenen. Conjugatie werd geanalyseerd d.m.v. uitplatingen en flow cytometrie. Voor 14 van de 15 geteste stammen werden er transconjuganten gedetecteerd. De transfer ratio, zijnde de verhouding van het aantal transconjuganten t.o.v. het totale celaantal, bleek afhankelijk te zijn van de acceptor stam en kon oplopen tot een grootteorde van 10^{-2}, m.a.w. 1 op 100 bacteriën verkregen het plasmide. Aan de hand van antibioticumgevoeligheidsprofielen van de acceptoren en de transconjuganten kon bevestigd worden dat de bacteriën na het verkrijgen van het plasmide resistentie vertoonden tegen bovenvermelde antibiotica.

Integrons zijn genetische elementen die vaak geassocieerd zijn met plasmiden. Ze zijn een mooi voorbeeld van de fascinerende wijze waarop bacteriën antibioticumresistentie kunnen verwerven en verder verspreiden. In Hoofdstuk 3 werd een Belgische collectie van Shiga-toxine producerende *E. coli* (STEC) gescreend op de aanwezigheid van integrons. STEC wordt beschouwd als de belangrijkste groep van opkomende voedselgebonden pathogenen. Integrons werden teruggevonden in 7.5% van de geanalyseerde stammen, allemaal van humane oorsprong. Ze behoorden allemaal tot klasse 1 integrons. Deze klasse is de meest voorkomende in Gram-negatieve bacteriën. Verdere karakterisering toonde aan dat de gedetecteerde integrons antibioticumresistentiegenen bevatten tegen twee types van antibiotica, namelijk tegen streptomycine/spectinomycine en tegen trimethoprim. Dit zou goed nieuws kunnen zijn, maar analyse van de antibioticumgevoeligheidsprofielen toonde aan dat 91.3% van de integron-positieve stammen resistentie vertoonde tegen minstens drie verschillende antibiotica. Van de integron-negatieve stammen daarentegen waren er 77.0% gevoelig voor al de geteste antibiotica.

In de voedingsindustrie kunnen biofilms een persisterende bron van contaminatie vormen, welke kan bijdragen aan voedselbederf, schade aan de apparatuur en een risico kan vormen voor de volksgezondheid indien pathogene bacteriën betrokken zijn. Bovendien worden deze structuren beschouwd als hotspots voor plasmidetransfer, waardoor zij kunnen bijdragen aan de verspreiding van antibioticumresistentie. In Hoofdstuk 4 werd de transfer van het multiresistent plasmide, pB10, nagegaan in biofilm modellen, representatief voor biofilms uit de voedingsindustrie. Hiervoor werd er gebruik gemaakt van twee verschillende vloeistofstroomconfiguraties (continu en druppelsgewijs) en drie aanhechtingsmaterialen (siliconen, glas en roestvrij staal). Net zoals in Hoofdstuk 2, werd er gewerkt met *P. putida* als donor en *E. coli* als acceptor. Als inoculatiestrategie werd er gekozen om eerst een biofilm te vormen met één van de twee bacteriesoorten en dan pas de tweede er op aan te brengen. Zo werd plasmidetransfer bestudeerd door middel van enerzijds een biofilm met plasmide donerende capaciteit en anderzijds een biofilm met plasmide ontvangende capaciteit.
Hoge transfer ratio's (aantal transconjuganten t.o.v. het totale celaantal) werden bekomen, die konden oplopen tot de grootteorde 10^{-1}.

Om voedsel gedurende voldoende tijd te kunnen bewaren wordt er gebruik gemaakt van bewaringstechnieken, die ervoor zorgen dat de bacteriën aanwezig op de voeding niet kunnen uitgroeien. Twee dergelijke technieken zijn lage temperatuur en het verpakken van

voedingsmiddelen onder gemodificeerde atmosfeer. In Hoofdstuk 5 werd het effect van deze twee technieken op plasmidetransfer in een Gram-positief model bestudeerd. Als donor werd er gebruik gemaakt van *Lactobacillus sakei* subsp. *sakei*, een typische Gram-positieve bederver, en als acceptor werd *Listeria monocytogenes*, een Gram-positieve psychrotrofe pathogeen, gebruikt. Beide soorten kunnen aangetroffen worden op kant-en-klare levensmiddelen, verpakt onder gemodificeerde atmosfeer (MAP). Wat temperatuur betreft, werd er plasmidetransfer geobserveerd in een range tussen 10 °C en 37 °C. De ondergrens kon echter wel verlaagd worden door de incubatieperiode te verlengen. Om het effect van gemodificeerde atmosfeer na te gaan werden er drie gassamenstellingen (lucht, 50% CO_2/50% N_2 en 100% N_2) toegepast. Wanneer er hoge startdensiteiten werden aangewend, werd plasmidetransfer onder elke conditie waargenomen. Dit zowel *in vitro*, op agarplaten, als *in situ*, op sneetjes gekookte ham. Om tot een realistischere situatie te komen, werd plasmidetransfer ook geanalyseerd op gekookte ham met lage startdensiteiten. Hierbij werd er enkel transfer waargenomen bij de 100% N_2 conditie na tien dagen incubatie. Onder deze conditie werd de hoogste bacteriële densiteit bekomen. Bij de MAP experimenten lag de transfer ratio, uitgedrukt als de verhouding transconjuganten t.o.v. het aantal acceptoren, steeds in de grootteorde $10^{-4} – 10^{-6}$. Hierbij dient er wel opgemerkt te worden dat transfer enkel werd waargenomen bij donor en acceptor densiteiten die de voedselveiligheidscriteria of -richtlijnen overschrijden. Indien men dus deze criteria/richtlijnen kan waarborgen, lijkt de bijdrage aan de verspreiding van antibioticumresistentie minimaal.

Het onderzoek uitgevoerd in dit doctoraat belichtte een klein aspect van de factoren betrokken bij de problematiek van de verspreiding van antibioticumresistentie. Niettemin werden er een aantal belangrijke bevindingen gedaan. Eerst en vooral werd er aangetoond dat de omgeving en de voeding geen strikt afgebakende niches zijn, maar dat deze ongetwijfeld kunnen interageren met elkaar. Ten tweede blijkt het toch van belang om integrons verder op te volgen, gezien zij vaak geassocieerd zijn met mobiele genetische elementen die bijkomende antibioticumresistentiegenen kunnen dragen. Verder werd duidelijk dat biofilms niet enkel een bron van contaminatie zijn in de voedingsindustrie, maar dat men ook op de hoede moet zijn voor het risico op verspreiding van antibioticumresistentie via plasmidetransfer in biofilms. Ten slotte bleken twee veel gebruikte bewaringstechnieken aangewend ter voorkoming van bacteriële groei in de voedingsindustrie, niet noodzakelijkerwijs plasmidetransfer te verhinderen.

Verder onderzoek met andere modelsystemen is echter noodzakelijk om onze kennis betreffende de rol die de voedselproductie en -bewaring speelt in de verspreiding van antibioticumresistentie te verruimen.

References

Aarestrup FM, Wegener HC, Collignon P (2008). Resistance in bacteria of the food chain: epidemiology and control strategies. *Expert Review of Anti-Infective Therapy* **6:** 733-750.

Aertsen A, Faster D, Michiels CW (2005). Induction of Shiga toxin-converting prophage in *Escherichia coli* by high hydrostatic pressure. *Applied and Environmental Microbiology* **71:** 1155-1162.

Ahmed AM, Younis EEA, Osman SA, Ishida Y, El-Khodety SA, Shimamoto T (2009). Genetic analysis of antimicrobial resistance in *Escherichia coli* isolated from diarrheic neonatal calves. *Veterinary Microbiology* **136:** 397-402.

Al-Nabulsi AA, Osaili TM, Elabedeen NAZ, Jaradat ZW, Shaker RR, Kheirallah KA, Tarazi YH, Holley RA (2011). Impact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of *Cronobacter sakazakii*. *International Journal of Food Microbiology* **146:** 137-143.

Alanis AJ (2005). Resistance to antibiotics: are we in the post-antibiotic era? *Archives of Medical Research* **36:** 697-705.

Alcaine SD, Warnick LD, Wiedmann M (2007). Antimicrobial resistance in nontyphoidal *Salmonella*. *Journal of Food Protection* **70:** 780-790.

Allen KJ, Wałecka-Zacharskaa E, Chen JC, Kosek-Paszkowska K, Devlieghere F, Van Meervenne E, Kovacevic J, Osek J, Wieczorek K & Bania J. *Listeria monocytogenes* - an examination of food chain factors potentially contributing to antimicrobial resistance. *Submitted to Food Microbiology.*

Ammor MS, Gueimonde M, Danielsen M, Zagorec M, van Hoek A, Reyes-Gavilán C, Mayo B, Margolles A (2008). Two different tetracycline resistance mechanisms, plasmid-carried *tet*(L) and chromosomally located transposon-associated *tet*(M), coexist in *Lactobacillus sakei* Rits 9. *Applied and Environmental Microbiology* **74:** 1394-1401.

Andersen JB, Sternberg C, Poulsen LK, Bjørn SP, Givskov M, Molin S (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Applied and Environmental Microbiology* **64:** 2240-2246.

Andersen TE, Kingshott P, Palarasah Y, Benter M, Alei M, Kolmos HJ (2010). A flow chamber assay for quantitative evaluation of bacterial surface colonization used to investigate the influence of temperature and surface hydrophilicity on the biofilm forming capacity of uropathogenic *Escherichia coli*. *Journal of Microbiological Methods* **81:** 135-140.

Angles ML, Marshall KC, Goodman AE (1993). Plasmid transfer between marine bacteria in the aqueous phase and biofilms in reactor microcosms. *Applied and Environmental Microbiology* **59:** 843-850.

Angulo FJ, Nargund VN, Chiller TC (2004). Evidence of an association between use of anti-microbial agents in food animals and anti-microbial resistance among bacteria isolated from humans and the human health consequences of such resistance. *Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health* **51:** 374-379.

Artés F, Allende A (2005). Processing lines and alternative preservation techniques to prolong the shelf-life of minimally fresh processed leafy vegetables. *European Journal of Horticultural Science* **70:** 231-245.

References

Arvanitoyannis IS, Stratakos AC (2012). Application of modified atmosphere packaging and active/smart technologies to red meat and poultry: a review. *Food and Bioprocess Technology* **5:** 1423-1446.

Audenaert K, D'Haene K, Messens K, Ruyssen T, Vandamme P, Huys G (2010). Diversity of lactic acid bacteria from modified atmosphere packaged sliced cooked meat products at sell-by date assessed by PCR-denaturing gradient gel electrophoresis. *Food Microbiology* **27:** 12-18.

Aune TEV, Aachmann FL (2010). Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. *Applied Microbiology and Biotechnology* **85:** 1301-1313.

Baharoglu Z, Bikard D, Mazel D (2010). Conjugative DNA transfer induces the bacterial SOS response and promotes antibiotic resistance development through integron activation. *Plos Genetics* **6:** e1001165.

Baharoglu Z, Krin E, Mazel D (2012). Connecting environment and genome plasticity in the characterization of transformation-induced SOS regulation and carbon catabolite control of the *Vibrio cholerae* integron integrase. *Journal of Bacteriology* **194:** 1659-1667.

Bahl MI, Burmølle M, Meisner A, Hansen LH, Sørensen SJ (2009). All IncP-1 plasmid subgroups, including the novel ε subgroup, are prevalent in the influent of a Danish wastewater treatment plant. *Plasmid* **62:** 134-139.

Bakkali M (2013). Could DNA uptake be a side effect of bacterial adhesion and twitching motility? *Archives of Microbiology* **195:** 279-289.

Barrangou R, Marraffini LA (2014). CRISPR-Cas Systems: Prokaryotes upgrade to adaptive immunity. *Mol Cell* **54:** 234-244.

Barlow M (2009). What antimicrobial resistance has taught us about horizontal gene transfer. *Methods in Molecular Biology* **532:** 397-411.

Barza M, Travers K (2002). Excess infections due to antimicrobial resistance: the "Attributable Fraction". *Clinical Infectious Diseases* **34 Suppl 3:** S126-130.

Bauer F, Hertel C, Hammes WP (1999). Transformation of *Escherichia coli* in foodstuffs. *Systematic and Applied Microbiology* **22:** 161-168.

Bauer T, Weller P, Hammes WP, Hertel C (2003). The effect of processing parameters on DNA degradation in food. *European Food Research and Technology* **217:** 338-343.

Bauer T, Hammes WP, Haase NU, Hertel C (2004). Effect of food components and processing parameters on DNA degradation in food. *Environmental Biosafety Research* **3:** 215-223.

Beceiro A, Tomás M, Bou G (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clinical Microbiology Reviews* **26:** 185-230.

Belgisch Staatsblad (2003). Koninklijk besluit betreffende autocontrole, meldingsplicht en traceerbaarheid in de voedselketen dd. 14 November 2003. *Belgisch Staatsblad* **431:** 59072-59086.

Ben Sallem R, Ben Slama K, Sáenz Y, Rojo-Bezares B, Estepa V, Jouini A, Gharsa H, Klibi N, Boudabous A, Torres C (2012). Prevalence and characterization of extended-spectrum beta-Lactamase (ESBL)- and CMY-2-producing *Escherichia coli* isolates from healthy food-producing animals in Tunisia. *Foodborne Pathogens and Disease* **9:** 1137-1142.

Bennett PM (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology* **153 Suppl 1:** S347-357.

Bernier SP, Lebeaux D, DeFrancesco AS, Valomon A, Soubigou G, Coppée JY, Ghigo JM, Beloin C (2013). Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. *Plos Genetics* **9:** e1003144.

Bertsch D, Uruty A, Anderegg J, Lacroix C, Perreten V, Meile L (2013). Tn*6198*, a novel transposon containing the trimethoprim resistance gene *dfrG* embedded into a Tn*916* element in *Listeria monocytogenes*. *Journal of Antimicrobial Chemotherapy* **68:** 986-991.

Beuls E, Modrie P, Deserranno C, Mahillon J (2012). High-salt stress conditions increase the pAW63 transfer frequency in *Bacillus thuringiensis*. *Applied and Environmental Microbiology* **78:** 7128-7131.

Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, Barton HA, Wright GD (2012). Antibiotic resistance is prevalent in an isolated cave microbiome. *Plos One* **7:** e34953.

Boerlin P, Travis R, Gyles CL, Reid-Smith R, Janecko N, Lim H, Nicholson V, McEwen SA, Friendship R, Archambault M (2005). Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *Applied and Environmental Microbiology* **71:** 6753-6761.

Boerlin P, Reid-Smith RJ (2008). Antimicrobial resistance: its emergence and transmission. *Animal Health Research Reviews* **9:** 115-126.

Bolton DJ (2011). Verocytotoxigenic (Shiga toxin-producing) *Escherichia coli*: virulence factors and pathogenicity in the farm to fork paradigm. *Foodborne Pathogens and Disease* **8:** 357-365.

Boon N, Goris J, De Vos P, Verstraete W, Top EM (2000). Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2*gfp*. *Applied and Environmental Microbiology* **66:** 2906-2913.

Boon N, Depuydt S, Verstraete W (2006). Evolutionary algorithms and flow cytometry to examine the parameters influencing transconjugant formation. *Fems Microbiology Ecology* **55:** 17-27.

Borch E, Kant-Muermans ML, Blixt Y (1996). Bacterial spoilage of meat and cured meat products. *International Journal of Food Microbiology* **33:** 103-120.

Bos R, van der Mei HC, Meinders JM, Busscher HJ (1994). A quantitative method to study co-adhesion of microorganisms in a parallel plate flow chamber: basic principles of the analysis. *Journal of Microbiological Methods* **20:** 289-305.

Botteldoorn N, Heyndrickx M, Rijpens N, Herman L (2003). Detection and characterization of verotoxigenic *Escherichia coli* by a VTEC/EHEC multiplex PCR in porcine faeces and pig carcass swabs. *Research in Microbiology* **154:** 97-104.

Boulares M, Mankai M, Aouadhi C, Olfa BM, Hassouna M (2013). Characterisation and identification of spoilage psychotrophic Gram-negative bacteria originating from Tunisian fresh fish. *Annals of Microbiology* **63:** 733-744.

Bowler LD, Zhang QY, Riou JY, Spratt BG (1994). Interspecies recombination between the *penA* genes of *Neisseria meningitidis* and commensal *Neisseria* species during the emergence of penicillin resistance in *N. meningitidis*: natural events and laboratory simulation. *Journal of Bacteriology* **176:** 333-337.

Box ATA, Mevius DJ, Schellen P, Verhoef J, Fluit AC (2005). Integrons in *Escherichia coli* from food-producing animals in the Netherlands. *Microbial Drug Resistance* **11:** 53-57.

Brabban AD, Hite E, Callaway TR (2005). Evolution of foodborne pathogens via temperate bacteriophage-mediated gene transfer. *Foodborne Pathogens and Disease* **2:** 287-303.

Bradley DE (1983). Specification of the conjugative pili and surface mating systems of *Pseudomonas* plasmids. *Journal of General Microbiology* **129:** 2545-2556.

Bradley DE, Taylor DE, Cohen DR (1980). Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. *Journal of Bacteriology* **143:** 1466-1470.

Bräutigam M, Hertel C, Hammes WP (1997). Evidence for natural transformation of *Bacillus subtilis* in foodstuffs. *Fems Microbiology Letters* **155:** 93-98.

Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F (2011). Resistance of bacterial biofilms to disinfectants: a review. *Biofouling* **27:** 1017-1032.

Brooks JD, Flint SH (2008). Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science and Technology* **43:** 2163-2176.

Bruun MS, Schmidt AS, Dalsgaard I, Larsen JL (2003). Conjugal transfer of large plasmids conferring oxytetracycline (OTC) resistance: Transfer between environmental aeromonads, fish-pathogenic bacteria, and *Escherichia coli*. *Journal of Aquatic Animal Health* **15:** 69-79.

Buchholz U, Bernard H, Werber D, Böhmer MM, Remschmidt C, Wilking H, Deleré Y, an der Heiden M, Adlhoch C, Dreesman J, Ehlers J, Ethelberg S, Faber M, Frank C, Fricke G, Greiner M, Höhle M, Ivarsson S, Jark U, Kirchner M, Koch J, Krause G, Luber P, Rosner B, Stark K, Kühne M (2011). German outbreak of *Escherichia coli* O104:H4 associated with sprouts. *The New England Journal of Medicine* **365:** 1763-1770.

Buckingham-Meyer K, Goeres DM, Hamilton MA (2007). Comparative evaluation of biofilm disinfectant efficacy tests. *Journal of Microbiological Methods* **70:** 236-244.

Burmølle M, Bahl MI, Jensen LB, Sørensen SJ, Hansen LH (2008). Type 3 fimbriae, encoded by the conjugative plasmid pOLA52, enhance biofilm formation and transfer frequencies in *Enterobacteriaceae* strains. *Microbiology* **154:** 187-195.

Burrus V, Pavlovic G, Decaris B, Guédon G (2002). Conjugative transposons: the tip of the iceberg. *Molecular Microbiology* **46:** 601-610.

Burrus V, Waldor MK (2004). Shaping bacterial genomes with integrative and conjugative elements. *Research in Microbiology* **155:** 376-386.

Buvens G, Bogaerts P, Glupczynski Y, Lauwers S, Piérard D (2010). Antimicrobial resistance testing of verocytotoxin-producing *Escherichia coli* and first description of TEM-52 extended-spectrum β-lactamase in serogroup O26. *Antimicrobial Agents and Chemotherapy* **54:** 4907-4909.

Buvens G, De Gheldre Y, Dediste A, de Moreau A-I, Mascart G, Simon A, Allemeersch D, Scheutz F, Lauwers S, Piérard D (2012). Incidence and virulence determinants of verocytotoxin-producing *Escherichia coli* infections in the Brussels-Capital Region, Belgium, in 2008-2010. *Journal of Clinical Microbiology* **50:** 1336-1345.

Byrd-Bredbenner C, Berning J, Martin-Biggers J, Quick V (2013). Food safety in home kitchens: A synthesis of the literature. *International Journal of Environmental Research and Public Health* **10:** 4060-4085.

Cabello FC (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology* **8:** 1137-1144.

Cabellos-Avelar T, Souza V, Membrillo-Hernández J (2006). Spent media from cultures of environmental isolates of *Escherichia coli* can suppress the deficiency of biofilm formation under anoxic conditions of laboratory *E. coli* strains. *Fems Microbiology Ecology* **58:** 414-424.

Caleb OJ, Mahajan PV, Al-Said FA, Opara UL (2013). Modified atmosphere packaging technology of fresh and fresh-cut produce and the microbial consequences - a review. *Food and Bioprocess Technology* **6:** 303-329.

Cambray G, Guerout AM, Mazel D (2010). Integrons. *Annual Review of Genetics* **44:** 141-166.

Campos J, Mourão J, Pestana N, Peixe L, Novais C, Antunes P (2013). Microbiological quality of ready-to-eat salads: an underestimated vehicle of bacteria and clinically relevant antibiotic resistance genes. *International Journal of Food Microbiology* **166:** 464-470.

Capita R, Alonso-Calleja C (2013). Antibiotic-resistant bacteria: a challenge for the food industry. *Critical Reviews in Food Science and Nutrition* **53:** 11-48.

Caplice E, Fitzgerald GF (1999). Food fermentations: role of microorganisms in food production and preservation. *International Journal of Food Microbiology* **50:** 131-149.

Cardoen S, Van Huffel X, Berkvens D, Quoilin S, Ducoffre G, Saegerman C, Speybroeck N, Imberechts H, Herman L, Ducatelle R, Dierick K (2009). Evidence-based semiquantitative methodology for prioritization of foodborne zoonoses. *Foodborne Pathogens and Disease* **6:** 1083-1096.

Carpenter RJ, Hartzell JD, Forsberg JA, Babel BS, Ganesan A (2008). *Pseudomonas putida* war wound infection in a US Marine: a case report and review of the literature. *Journal of Infection* **56:** 234-240.

Casamayor EO, Schäfer H, Bañeras L, Pedrós-Alió C, Muyzer G (2000). Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology* **66:** 499-508.

References

Casiraghi E, Alamprese C, Pompei C (2007). Cooked ham classification on the basis of brine injection level and pork breeding country. *Lwt-Food Science and Technology* **40:** 164-169.

Castellano P, Belfiore C, Fadda S, Vignolo G (2008). A review of bacteriocinogenic lactic acid bacteria used as bioprotective cultures in fresh meat produced in Argentina. *Meat Science* **79:** 483-499.

Castonguay MH, van der Schaaf S, Koester W, Krooneman J, van der Meer W, Harmsen H, Landini P (2006). Biofilm formation by *Escherichia coli* is stimulated by synergistic interactions and co-adhesion mechanisms with adherence-proficient bacteria. *Research in Microbiology* **157:** 471-478.

Cergole-Novella MC, Pignatari ACC, Castanheira M, Guth BEC (2011). Molecular typing of antimicrobial-resistant Shiga-toxin-producing *Escherichia coli* strains (STEC) in Brazil. *Research in Microbiology* **162:** 117-123.

Chaillou S, Lucquin I, Najjari A, Zagorec M, Champomier-Vergès MC (2013). Population genetics of *Lactobacillus sakei* reveals three lineages with distinct evolutionary histories. *Plos One* **8:** e73253.

Chaisatit C, Tribuddharat C, Pulsrikarn C, Dejsirilert S (2012). Molecular characterization of antibiotic-resistant bacteria in contaminated chicken meat sold at supermarkets in Bangkok, Thailand. *Japanese Journal of Infectious Diseases* **65:** 527-534.

Champomier-Vergès MC, Chaillou S, Cornet M, Zagorec M (2002). Erratum to "*Lactobacillus sakei*: recent developments and future prospects" [Research in Microbiology 152 (2001) 839]. *Research in Microbiology* **153:** 115-123.

Chan YC, Wiedmann M (2009). Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. *Critical Reviews in Food Science and Nutrition* **49:** 237-253.

Chapman JS (2003). Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *International Biodeterioration & Biodegradation* **51:** 271-276.

Chellappa ST, Maredia R, Phipps K, Haskins WE, Weitao T (2013). Motility of *Pseudomonas aeruginosa* contributes to SOS-inducible biofilm formation. *Research in Microbiology* **164:** 1019-1027.

Chen I, Christie PJ, Dubnau D (2005). The ins and outs of DNA transfer in bacteria. *Science* **310:** 1456-1460.

Chiou CS, Jones AL (1995). Expression and identification of the *strA-strB* gene pair from streptomycin-resistant *Erwinia amylovora*. *Gene* **152:** 47-51.

Chmielewski RAN, Frank JF (2003). Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety* **2:** 22-32.

Christensen BB, Sternberg C, Andersen JB, Eberl L, Møller S, Givskov M, Molin S (1998). Establishment of new genetic traits in a microbial biofilm community. *Applied and Environmental Microbiology* **64:** 2247-2255.

Church N (1994). Developments in modified-atmosphere packaging and related technologies. *Trends in Food Science & Technology* **5:** 345-352.

Church IJ, Parsons AL (1995). Modified atmosphere packaging technology: a review. *Journal of the Science of Food and Agriculture* **67:** 143-152.

Cloete E, Molobela I, Van Der Merwe A, Richards M (2009). Biofilms in the food and beverage industries: an introduction. In Fratamico PM, Annous BA, Gunther NW (Eds.) *Biofilms in the food and beverage industries.* Cambridge, UK, Woodhead Publishing Limited.

CLSI (2009). Performance standards for antimicrobial susceptibility testing, *CLSI document M02-A10*, Wayne, USA, Clinical and Laboratory Standards Institute.

Cocconcelli PS, Cattivelli D, Gazzola S (2003). Gene transfer of vancomycin and tetracycline resistances among *Enterococcus faecalis* during cheese and sausage fermentations. *International Journal of Food Microbiology* **88:** 315-323.

Coenye T, Nelis HJ (2010). *In vitro* and *in vivo* model systems to study microbial biofilm formation. *Journal of Microbiological Methods* **83:** 89-105.

Collignon P (2013). Superbugs in food: a severe public health concern. *Lancet Infectious Diseases* **13:** 641-643.

Collignon P, Aarestrup FM, Irwin R, McEwen S (2013). Human deaths and third-generation cephalosporin use in poultry, Europe. *Emerging Infectious Diseases* **19:** 1339-1340.

Colón-González M, Méndez-Ortiz MM, Membrillo-Hernández J (2004). Anaerobic growth does not support biofilm formation in *Escherichia coli* K-12. *Research in Microbiology* **155:** 514-521.

Cotter PD, Hill C, Ross RP (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology* **3:** 777-788.

D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006). Sampling the antibiotic resistome. *Science* **311:** 374-377.

D'Costa VM, King CE, Kalan L, Morar M, Sung WW, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD (2011). Antibiotic resistance is ancient. *Nature* **477:** 457-461.

Dalton HM, Poulsen LK, Halasz P, Angles ML, Goodman AE, Marshall KC (1994). Substratum-induced morphological changes in a marine bacterium and their relevance to biofilm structure. *Journal of Bacteriology* **176:** 6900-6906.

Davin-Regli A, Pagès JM (2012). Cross-resistance between biocides and antimicrobials: an emerging question. *Revue Scientifique et Technique - Office International des Epizooties* **31:** 89-104.

De Gelder L, Vandecasteele FPJ, Brown CJ, Forney LJ, Top EM (2005). Plasmid donor affects host range of promiscuous IncP-1β plasmid pB10 in an activated-sludge microbial community. *Applied and Environmental Microbiology* **71:** 5309-5317.

de Kraker ME, Davey PG, Grundmann H (2011). Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteremia: estimating the burden of antibiotic resistance in Europe. *PLoS Medicine* **8:** e1001104.

de la Fuente-Núñez C, Reffuveille F, Fernández L, Hancock REW (2013). Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Current Opinion in Microbiology* **16:** 580-589.

De Roy K, Clement L, Thas O, Wang Y, Boon N (2012). Flow cytometry for fast microbial community fingerprinting. *Water Research* **46:** 907-919.

References

de Vries J, Wackernagel W (2004). Microbial horizontal gene transfer and the DNA release from transgenic crop plants. *Plant and Soil* **266:** 91-104.

Defoirdt T, Boon N, Bossier P (2010). Can bacteria evolve resistance to quorum sensing disruption? *Plos Pathogens* **6:** e1000989.

Devirgiliis C, Barile S, Perozzi G (2011). Antibiotic resistance determinants in the interplay between food and gut microbiota. *Genes and Nutrition* **6:** 275-284.

Devirgiliis C, Zinno P, Perozzi G (2013). Update on antibiotic resistance in foodborne *Lactobacillus* and *Lactococcus* species. *Frontiers in Microbiology* **4:** 301.

Di Luca MC, D'Ercole S, Petrelli D, Prenna M, Ripa S, Vitali LA (2010). Lysogenic transfer of *mef*(A) and *tet*(O) genes carried by Φm46.1 among group A streptococci. *Antimicrobial Agents and Chemotherapy* **54:** 4464-4466.

Dibner JJ, Richards JD (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science* **84:** 634-643.

Domingues S, da Silva GJ, Nielsen KM (2012). Integrons: vehicles and pathways for horizontal dissemination in bacteria. *Mob Genet Elements* **2:** 211-223.

Donlan RM, Costerton JW (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* **15:** 167-193.

Doublet B, Boyd D, Mulvey MR, Cloeckaert A (2005). The *Salmonella* genomic island 1 is an integrative mobilizable element. *Molecular Microbiology* **55:** 1911-1924.

Doulgeraki AI, Nychas GJE (2013). Monitoring the succession of the biota grown on a selective medium for pseudomonads during storage of minced beef with molecular-based methods. *Food Microbiology* **34:** 62-69.

Drenkard E (2003). Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes and Infection* **5:** 1213-1219.

Drevets DA, Bronze MS (2008). *Listeria monocytogenes*: epidemiology, human disease, and mechanisms of brain invasion. *Fems Immunology and Medical Microbiology* **53:** 151-165.

Dröge M, Pühler A, Selbitschka W (2000). Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Molecular and General Genetics* **263:** 471-482.

Dubnau D (1999). DNA uptake in bacteria. *Annual Review of Microbiology* **53:** 217-244.

Dudley EG, Abe C, Ghigo JM, Latour-Lambert P, Hormazabal JC, Nataro JP (2006). An IncI1 plasmid contributes to the adherence of the atypical enteroaggregative *Escherichia coli* strain C1096 to cultured cells and abiotic surfaces. *Infection and Immunity* **74:** 2102-2114.

Dupuis ME, Villion M, Magadán AH, Moineau S (2013). CRISPR-Cas and restriction-modification systems are compatible and increase phage resistance. *Nature Communications* **4:** 2087.

ECDC/EMEA (2009). ECDC/EMEA Joint technical report. The bacterial challenge: time to react. Available at: http://www.ecdc.europa.eu/en/publications/_layouts/forms/Publication_DispForm.aspx?ID=199&List=4f55ad51-4aed-4d32-b960-af70113dbb90. Date last accessed: April 12, 2014.

ECDC (2013). Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Available at: http://www.ecdc.europa.eu/en/publications/_layouts/forms/Publication_DispForm.aspx?List=4f55ad51-4aed-4d32-b960-af70113dbb90&ID=963. Date last accessed: June 5, 2014.

EFSA (2006). The community summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks in the European Union in 2005. *The EFSA Journal* **94:** 288pp.

EFSA (2007). Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *The EFSA Journal* **587:** 1-16.

EFSA/ECDC (2007). The community summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks in the European Union in 2006. *The EFSA Journal* **130:** 352pp.

EFSA (2008). Scientific Opinion of the Panel on Biological Hazards on a request from the European Food Safety Authority on foodborne antimicrobial resistance as a biological hazard. *The EFSA Journal* **765:** 1-87.

EFSA (2009). Scientific Opinion of the Panel on Biological Hazards on a request from European Commission on Quantitative estimation of the impact of setting a new target for the reduction of *Salmonella* in breeding hens of *Gallus gallus*. *The EFSA Journal* **1036:** 1-68.

EFSA/ECDC (2009a). The community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. *The EFSA Journal* 223: 215pp.

EFSA/ECDC (2009b). The community summary report on food-borne outbreaks in the European Union in 2007. *The EFSA Journal* **271:** 128pp.

EFSA/ECDC (2010). The community summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the European Union in 2008. *EFSA Journal* **8:** 1496 [368pp].

EFSA/ECDC (2011). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2009. *EFSA Journal* **9:** 2090 [378pp].

EFSA/ECDC (2012). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. *EFSA Journal* **10:** 2597 [442pp].

EFSA (2012). Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). *EFSA Journal* **10:** 3020 [84pp.].

EFSA/ECDC (2013). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. *EFSA Journal* **11:** 3129 [250pp.].

EFSA (2013). Analysis of the baseline survey on the prevalence of *Listeria monocytogenes* in certain ready-to-eat (RTE) foods in the EU, 2010-2011 - Part A: *Listeria monocytogenes* prevalence estimates. *EFSA Journal* **11:** 3241 [75pp].

References

EFSA/ECDC (2014). The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2012. *EFSA Journal* **12:**3590 [336pp.]

Erill I, Campoy S, Barbe J (2007). Aeons of distress: an evolutionary perspective on the bacterial SOS response. *Fems Microbiology Reviews* **31:** 637-656.

Fajardo A, Martínez-Martín N, Mercadillo M, Galán JC, Ghysels B, Matthijs S, Cornelis P, Wiehlmann L, Tümmler B, Baquero F, Martínez JL (2008). The neglected intrinsic resistome of bacterial pathogens. *Plos One* **3:** e1619.

FAO/OIE/WHO (2003). Joint FAO/OIE/WHO Expert workshop on non-human antimicrobial usage and antimicrobial resistance: Scientific assessment Geneva, December 1 – 5, 2003. Available at: http://www.who.int/foodsafety/micro/meetings/nov2003/en/ Date last accessed: April 11, 2014.

FAO/WHO (2007). Guidance to governments on the application of HACCP in small and/or less-developed food businesses. *FAO Food and Nutrition Paper* **86:** 92pp.

Farber JM (1991). Microbiological aspects of modified-atmosphere packaging technology - a review. *Journal of Food Protection* **54:** 58-70.

Farkas J (2007) Physical methods of food preservation. In Doyle MP, Beuchat LR (Eds.) *Food Microbiology - Fundamentals and Frontiers.* Washington, USA, ASM Press.

Feiner G (2006) *Meat products handbook - Practical science and technology*. Woodhead Publishing Limited and CRC Press, Cambridge, UK.

Feliciano L, Lee J, Lopes JA, Pascall MA (2010). Efficacy of sanitized ice in reducing bacterial load on fish fillet and in the water collected from the melted ice. *Journal of Food Science* **75:** M231-238.

Fernandez-Astorga A, Muela A, Cisterna R, Iriberri J, Barcina I (1992). Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. *Applied and Environmental Microbiology* **58:** 392-398.

Finley RL, Collignon P, Larsson DGJ, McEwen SA, Li XZ, Gaze WH, Reid-Smith R, Timinouni M, Graham DW, Topp E (2013). The scourge of antibiotic resistance: the important role of the environment. *Clinical Infectious Diseases* **57:** 704-710.

Flamm RK, Hinrichs DJ, Thomashow MF (1984). Introduction of pamβ1 into *Listeria Monocytogenes* by conjugation and homology between native *L. monocytogenes* plasmids. *Infection and Immunity* **44:** 157-161.

Franiczek R, Krzyżanowska B, Dolna I, Mokracka-Latajka G (2010). Conjugative transfer of plasmid-mediated CTX-M-type beta-lactamases from clinical strains of *Enterobacteriaceae* to *Salmonella enterica* serovars. *Advances in Clinical and Experimental Medicine* **19:** 313-322.

Frank C, Werber D, Cramer JP, Askar M, Faber M, an der Heiden M, Bernard H, Fruth A, Prager R, Spode A, Wadl M, Zoufaly A, Jordan S, Kemper MJ, Follin P, Müller L, King LA, Rosner B, Buchholz U, Stark K, Krause G (2011). Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. *The New England Journal of Medicine* **365:** 1771-1780.

Ganjian H, Nikokar I, Tieshayar A, Mostafaei A, Amirmozafari N, Kiani S (2012). Effects of salt stress on the antimicrobial drug resistance and protein profile of *Staphylococcus aureus*. *Jundishapur Journal of Microbiology* **5:** 328-331.

García-Almendárez BE, Cann IKO, Martin SE, Guerrero-Legarreta I, Regalado C (2008). Effect of *Lactococcus lactis* UQ2 and its bacteriocin on *Listeria monocytogenes* biofilms. *Food Control* **19:** 670-680.

Garcillán-Barcia MP, Francia MV, de la Cruz F (2009). The diversity of conjugative relaxases and its application in plasmid classification. *Fems Microbiology Reviews* **33:** 657-687.

Gazzola S, Fontana C, Bassi D, Cocconcelli PS (2012). Assessment of tetracycline and erythromycin resistance transfer during sausage fermentation by culture-dependent and -independent methods. *Food Microbiology* **30:** 348-354.

Gevers D, Danielsen M, Huys G, Swings J (2003a). Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. *Applied and Environmental Microbiology* **69:** 1270-1275.

Gevers D, Huys G, Swings J (2003b). In vitro conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. *Fems Microbiology Letters* **225:** 125-130.

Ghanbari M, Jami M, Domig KJ, Kneifel W (2013). Seafood biopreservation by lactic acid bacteria - a review. *Lwt-Food Science and Technology* **54:** 315-324.

Ghigo JM (2001). Natural conjugative plasmids induce bacterial biofilm development. *Nature* **412:** 442-445.

Giammanco GM, Pignato S, Mammina C, Grimont F, Grimont PA, Nastasi A, Giammanco G (2002). Persistent endemicity of *Salmonella bongori* 48:z$_{35}$:- in Southern Italy: molecular characterization of human, animal, and environmental isolates. *Journal of Clinical Microbiology* **40:** 3502-3505.

Giaouris E, Heir E, Hébraud M, Chorianopoulos N, Langsrud S, Møretrø T, Habimana O, Desvaux M, Renier S, Nychas GJ (2014). Attachment and biofilm formation by foodborne bacteria in meat processing environments: causes, implications, role of bacterial interactions and control by alternative novel methods. *Meat Science* **97:** 298-309.

Gilbert ES, Walker AW, Keasling JD (2003). A constructed microbial consortium for biodegradation of the organophosphorus insecticide parathion. *Applied Microbiology and Biotechnology* **61:** 77-81.

Gillings MR (2014). Integrons: Past, Present, and Future. *Microbiology and Molecular Biology Reviews* **78:** 257-277.

Glenn LM, Englen MD, Lindsey RL, Frank JF, Turpin JE, Berrang ME, Meinersmann RJ, Fedorka-Cray PJ, Frye JG (2012). Analysis of antimicrobial resistance genes detected in multiple-drug-resistant *Escherichia coli* isolates from broiler chicken carcasses. *Microbial Drug Resistance* **18:** 453-463.

Goeres DM, Hamilton MA, Beck NA, Buckingham-Meyer K, Hilyard JD, Loetterle LR, Lorenz LA, Walker DK, Stewart PS (2009). A method for growing a biofilm under low shear at the air-liquid interface using the drip flow biofilm reactor. *Nature Protocols* **4:** 783-788.

Goessweiner-Mohr N, Arends K, Keller W, Grohmann E (2013). Conjugative type IV secretion systems in Gram-positive bacteria. *Plasmid* **70:** 289-302.

Goh S, Hussain H, Chang BJ, Emmett W, Riley TV, Mullany P (2013). Phage ΦC2 mediates transduction of Tn*6215*, encoding erythromycin resistance, between *Clostridium difficile* strains. *MBio* **4:** e00840-13.

Gómez NC, Abriouel H, Grande MJ, Pulido RP, Gálvez A (2012). Effect of enterocin AS-48 in combination with biocides on planktonic and sessile *Listeria monocytogenes*. *Food Microbiology* **30:** 51-58.

Goulter RM, Gentle IR, Dykes GA (2009). Issues in determining factors influencing bacterial attachment: a review using the attachment of *Escherichia coli* to abiotic surfaces as an example. *Letters in Applied Microbiology* **49:** 1-7.

Griffith, C. J. (2006) Food safety: where from and where to? *British Food Journal* **108** (1)**,** 6-15.

Grohmann E, Muth G, Espinosa M (2003). Conjugative plasmid transfer in Gram-positive bacteria. *Microbiology and Molecular Biology Reviews* **67:** 277-301.

Guerin E, Cambray G, Sanchez-Alberola N, Campoy S, Erill I, Da Re S, Gonzalez-Zorn B, Barbé J, Ploy MC, Mazel D (2009). The SOS response controls integron recombination. *Science* **324:** 1034.

Guerra B, Junker E, Schroeter A, Malorny B, Lehmann S, Helmuth R (2003). Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. *Journal of Antimicrobial Chemotherapy* **52:** 489-492.

Guillet C, Join-Lambert O, Le Monnier A, Leclercq A, Mechaï F, Mamzer-Bruneel MF, Bielecka MK, Scortti M, Disson O, Berche P, Vazquez-Boland J, Lortholary O, Lecuit M (2010). Human listeriosis caused by *Listeria ivanovii*. *Emerging Infectious Diseases* **16:** 136-138.

Gunasekera TS, Sørensen A, Attfield PV, Sørensen SJ, Veal DA (2002). Inducible gene expression by nonculturable bacteria in milk after pasteurization. *Applied and Environmental Microbiology* **68:** 1988-1993.

Gunduz GT, Tuncel G (2006). Biofilm formation in an ice cream plant. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **89:** 329-336.

Hächler H, Marti G, Giannini P, Lehner A, Jost M, Beck J, Weiss F, Bally B, Jermini M, Stephan R, Baumgartner A (2013). Outbreak of listerosis due to imported cooked ham, Switzerland 2011. *Eurosurveillance* **18:** 20469.

Hamer DH, Gill CJ (2002). From the farm to the kitchen table: the negative impact of antimicrobial use in animals on humans. *Nutrition Reviews* **60:** 261-264.

Hammad AM, Ishida Y, Shimamoto T (2009). Prevalence and molecular characterization of ampicillin-resistant *Enterobacteriaceae* isolated from traditional Egyptian Domiati cheese. *Journal of Food Protection* **72:** 624-630.

Hansen MC, Palmer RJ, Jr., Udsen C, White DC, Molin S (2001). Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology* **147:** 1383-1391.

Haug MC, Tanner SA, Lacroix C, Stevens MJ, Meile L (2011). Monitoring horizontal antibiotic resistance gene transfer in a colonic fermentation model. *Fems Microbiology Ecology*.

Hawkey PM, Jones AM (2009). The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy* **64:** 3-10.

He H, Dong J, Lee CN, Li Y (2009). Molecular analysis of spoilage-related bacteria in pasteurized milk during refrigeration by PCR and denaturing gradient gel electrophoresis. *Journal of Food Protection* **72:** 572-577.

Hegstad K, Langsrud S, Lunestad BT, Scheie AA, Sunde M, Yazdankhah SP (2010). Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? *Microbial Drug Resistance* **16:** 91-104.

Hennequin C, Aumeran C, Robin F, Traore O, Forestier C (2012). Antibiotic resistance and plasmid transfer capacity in biofilm formed with a CTX-M-15-producing *Klebsiella pneumoniae* isolate. *Journal of Antimicrobial Chemotherapy* **67:** 2123-2130.

Hilbert F, Bauer A, Bolton D (2012). Antibiotic resistance in asymptomatic commensal and shiga toxin-producing *Escherichia coli* (STEC). *Wiener Tierarztliche Monatsschrift* **99:** 306-314.

Ho PL, Wong RC, Chow KH, Que TL (2009). Distribution of integron-associated trimethoprim-sulfamethoxazole resistance determinants among *Escherichia coli* from humans and food-producing animals. *Letters in Applied Microbiology* **49:** 627-634.

Horvath P, Barrangou R (2010). CRISPR/Cas, the immune system of Bacteria and Archaea. *Science* **327:** 167-170.

IFT (2006). Antimicrobial resistance: implications for the food system. *Comprehensive Reviews in Food Science and Food Safety* **5:** 71-137.

Imamovic L, Jofre J, Schmidt H, Serra-Moreno R, Muniesa M (2009). Phage-mediated Shiga toxin 2 gene transfer in food and water. *Applied and Environmental Microbiology* **75:** 1764-1768.

Jacxsens L, Devlieghere F, Uyttendaele M (2009). *Quality management systems in the food industry*. UGent, Ghent, Belgium.

Jahid IK, Ha SD (2012). A review of microbial biofilms of produce: Future challenge to food safety. *Food Science and Biotechnology* **21:** 299-316.

Janoir C, Podglajen I, Kitzis MD, Poyart C, Gutmann L (1999). In vitro exchange of fluoroquinolone resistance determinants between *Streptococcus pneumoniae* and viridans streptococci and genomic organization of the *parE-parC* region in *S. mitis*. *The Journal of Infectious Diseases* **180:** 555-558.

Jay JM, Loessner MJ, Golden DA (2005). *Modern food microbiology*. Springer Science+Business Media, Inc., New York, USA.

Jayas DS, Jeyamkondan S (2002). Modified atmosphere storage of grains meats fruits and vegetables. *Biosystems Engineering* **82:** 235-251.

Jeon B, Muraoka W, Sahin O, Zhang Q (2008). Role of Cj1211 in natural transformation and transfer of antibiotic resistance determinants in *Campylobacter jejuni*. *Antimicrobial Agents and Chemotherapy* **52:** 2699-2708.

Johnsborg O, Eldholm V, Håvarstein LS (2007). Natural genetic transformation: prevalence, mechanisms and function. *Research in Microbiology* **158:** 767-778.

Jones T, Gill CO, McMullen LM (2004). The behaviour of log phase Escherichia coli at temperatures that fluctuate about the minimum for growth. *Letters in Applied Microbiology* **39:** 296-300.

Kang HY, Jeong YS, Oh JY, Tae SH, Choi CH, Moon DC, Lee WK, Lee YC, Seol SY, Cho DT, Lee JC (2005). Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. *Journal of Antimicrobial Chemotherapy* **55:** 639-644.

Karama M, Gyles CL (2010). Methods for genotyping verotoxin-producing *Escherichia coli*. *Zoonoses and Public Health* **57:** 447-462.

Kardos N, Demain AL (2011). Penicillin: the medicine with the greatest impact on therapeutic outcomes. *Applied Microbiology and Biotechnology* **92:** 677-687.

Karmali MA, Gannon V, Sargeant JM (2010). Verocytotoxin-producing *Escherichia coli* (VTEC). *Veterinary Microbiology* **140:** 360-370.

Katribe E, Bogomolnaya LM, Wingert H, Andrews-Polymenis H (2009). Subspecies IIIa and IIIb Salmonellae are defective for colonization of murine models of salmonellosis compared to *Salmonella enterica* subsp I serovar Typhimurium. *Journal of Bacteriology* **191:** 2843-2850.

Keese P (2008). Risks from GMOs due to horizontal gene transfer. *Environmental Biosafety Research* **7:** 123-149.

Kessler B, de Lorenzo V, Timmis KN (1992). A general system to integrate lacZ fusions into the chromosomes of gram-negative eubacteria: regulation of the Pm promoter of the TOL plasmid studied with all controlling elements in monocopy. *Molecular and General Genetics* **233:** 293-301.

Khaitsa ML, Oloya J, Doetkott D, Kegode R (2008). Antimicrobial resistance and association with class 1 integrons in *Escherichia coli* isolated from turkey meat products. *Journal of Food Protection* **71:** 1679-1684.

Kharazmi M, Bauer T, Hammes WP, Hertel C (2003). Effect of food processing on the fate of DNA with regard to degradation and transformation capability in *Bacillus subtilis*. *Systematic and Applied Microbiology* **26:** 495-501.

Kilonzo-Nthenge A, Chen FC, Godwin SL (2008). Occurrence of *Listeria* and *Enterobacteriaceae* in domestic refrigerators. *Journal of Food Protection* **71:** 608-612.

Kilonzo-Nthenge A, Rotich E, Godwin S, Nahashon S, Chen F (2012). Prevalence and antimicrobial resistance of *Cronobacter sakazakii* isolated from domestic kitchens in Middle Tennessee, United States. *Journal of Food Protection* **75:** 1512-1517.

Klein J, Altenbuchner J, Mattes R (1998). Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets. *Journal of Biotechnology* **60:** 145-153.

Köhler CD, Dobrindt U (2011). What defines extraintestinal pathogenic *Escherichia coli*? *International Journal of Medical Microbiology* **301:** 642-647.

Koraimann G (2004) Bacterial conjugation: cell-cell contact-dependent horizontal gene spread. In Miller RV, Day MJ. (Eds.) *Microbial evolution: gene establishment, survival, and exchange.* Washington, USA, ASM Press.

Król JE, Rogers LM, Krone SM, Top EM (2010). Dual reporter system for *in situ* detection of plasmid transfer under aerobic and anaerobic conditions. *Applied and Environmental Microbiology* **76:** 4553-4556.

Król JE, Nguyen HD, Rogers LM, Beyenal H, Krone SM, Top EM (2011). Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air-liquid interface. *Applied and Environmental Microbiology* **77:** 5079-5088.

Kruse H, Sørum H (1994). Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. *Applied and Environmental Microbiology* **60:** 4015-4021.

Kulp A, Kuehn MJ (2010). Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annual Review of Microbiology* **64:** 163-184.

Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infectious Diseases* **10:** 597-602.

Lampkowska J, Feld L, Monaghan A, Toomey N, Schjørring S, Jacobsen B, van der Voet H, Andersen SR, Bolton D, Aarts H, Krogfelt KA, Wilcks A, Bardowski J (2008). A standardized conjugation protocol to asses antibiotic resistance transfer between lactococcal species. *International Journal of Food Microbiology* **127:** 172-175.

Lang AS, Zhaxybayeva O, Beatty JT (2012). Gene transfer agents: phage-like elements of genetic exchange. *Nature Reviews Microbiology* **10:** 472-482.

Lapierre L, Cornejo J, Borie C, Toro C, San Martín B (2008). Genetic characterization of antibiotic resistance genes linked to class 1 and class 2 integrons in commensal strains of *Escherichia coli* isolated from poultry and swine. *Microbial Drug Resistance* **14:** 265-272.

Leistner L (2000). Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology* **55:** 181-186.

Levy-Booth DJ, Campbell RG, Gulden RH, Hart MM, Powell JR, Klironomos JN, Pauls KP, Swanton CJ, Trevors JT, Dunfield KE (2007). Cycling of extracellular DNA in the soil environment. *Soil Biology & Biochemistry* **39:** 2977-2991.

Lewis K (2010). Persister Cells. *Annual Review of Microbiology* **64:** 357-372.

Li MC, Wang F, Li F (2011). Identification and molecular characterization of antimicrobial-resistant Shiga toxin-producing *Escherichia coli* isolated from retail meat products. *Foodborne Pathogens and Disease* **8:** 489-493.

Lilley AK, Bailey MJ (2002). The transfer dynamics of *Pseudomonas* sp. plasmid pQBR11 in biofilms. *Fems Microbiology Ecology* **42:** 243-250.

Lorenz MG, Wackernagel W (1994). Bacterial gene transfer by natural genetic transformation in the environment. *Microbiological Reviews* **58:** 563-602.

References

Lungu B, O'Bryan CA, Muthaiyan A, Milillo SR, Johnson MG, Crandall PG, Ricke SC (2011). *Listeria monocytogenes*: antibiotic resistance in food production. *Foodborne Pathogens and Disease* **8:** 569-578.

Luo HL, Wan K, Wang HH (2005). High-frequency conjugation system facilitates biofilm formation and pAMβ1 transmission by *Lactococcus lactis*. *Applied and Environmental Microbiology* **71:** 2970-2978.

Lyautey E, Lacoste B, Ten-Hage L, Rols JL, Garabetian F (2005). Analysis of bacterial diversity in river biofilms using 16S rDNA PCR-DGGE: methodological settings and fingerprints interpretation. *Water Research* **39:** 380-388.

Macinga DR, Rather PN (1999). The chromosomal 2'-N-acetyltransferase of *Providencia stuartii*: physiological functions and genetic regulation. *Frontiers in Bioscience* **4:** D132-140.

Madsen JS, Burmølle M, Hansen LH, Sørensen SJ (2012). The interconnection between biofilm formation and horizontal gene transfer. *Fems Immunology and Medical Microbiology* **65:** 183-195.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* **18:** 268-281.

Mah TFC, O'Toole GA (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* **9:** 34-39.

Mahillon J, Chandler M (1998). Insertion sequences. *Microbiology and Molecular Biology Reviews* **62:** 725-774.

Manges AR, Smith SP, Lau BJ, Nuval CJ, Eisenberg JN, Dietrich PS, Riley LW (2007). Retail meat consumption and the acquisition of antimicrobial resistant *Escherichia coli* causing urinary tract infections: a case-control study. *Foodborne Pathogens and Disease* **4:** 419-431.

Manges AR, Johnson JR (2012). Food-borne origins of *Escherichia coli* causing extraintestinal infections. *Clinical Infectious Diseases* **55:** 712-719.

Manning AJ, Kuehn MJ (2013). Functional advantages conferred by extracellular prokaryotic membrane vesicles. *Journal of Molecular Microbiology and Biotechnology* **23:** 131-141.

Marchand S, De Block J, De Jonghe V, Coorevits A, Heyndrickx M, Herman L (2012). Biofilm formation in milk production and processing environments; influence on milk quality and safety. *Comprehensive Reviews in Food Science and Food Safety* **11:** 133-147.

Marchant M, Vinué L, Torres C, Moreno MA (2013). Change of integrons over time in *Escherichia coli* isolates recovered from healthy pigs and chickens. *Veterinary Microbiology* **163:** 124-132.

Marraffini LA, Sontheimer EJ (2008). CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* **322:** 1843-1845.

Marshall BM, Levy SB (2011). Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews* **24:** 718-733.

Marshall BM, Robleto E, Dumont T, Levy SB (2012). The frequency of antibiotic-resistant bacteria in homes differing in their use of surface antibacterial agents. *Current Microbiology* **65:** 407-415.

Martinez JL (2009). The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proceedings of the Royal Society B: Biological Sciences* **276:** 2521-2530.

Martinez JL, Sánchez MB, Martínez-Solano L, Hernandez A, Garmendia L, Fajardo A, Alvarez-Ortega C (2009). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *Fems Microbiology Reviews* **33:** 430-449.

Mathur S, Singh R (2005). Antibiotic resistance in food lactic acid bacteria - a review. *International Journal of Food Microbiology* **105:** 281-295.

Mazel D (2006). Integrons: agents of bacterial evolution. *Nature Reviews Microbiology* **4:** 608-620.

McDermott PF, Walker RD, White DG (2003). Antimicrobials: modes of action and mechanisms of resistance. *International Journal of Toxicology* **22:** 135-143.

McGannon CM, Fuller CA, Weiss AA (2010). Different classes of antibiotics differentially influence Shiga toxin production. *Antimicrobial Agents and Chemotherapy* **54:** 3790-3798.

Mc Mahon MAS, Blair IS, Moore JE, Mc Dowell DA (2007a). The rate of horizontal transmission of antibiotic resistance plasmids is increased in food preservation-stressed bacteria. *Journal of Applied Microbiology* **103:** 1883-1888.

McMahon MAS, Xu JR, Moore JE, Blair IS, McDowell DA (2007b). Environmental stress and antibiotic resistance in food-related pathogens. *Applied and Environmental Microbiology* **73:** 211-217.

Mejlholm O, Kjeldgaard J, Modberg A, Vest MB, Bøknæs N, Koort J, Björkroth J, Dalgaard P (2008). Microbial changes and growth of *Listeria monocytogenes* during chilled storage of brined shrimp (*Pandalus borealis*). *International Journal of Food Microbiology* **124:** 250-259.

Mejlholm O, Devitt TD, Dalgaard P (2012). Effect of brine marination on survival and growth of spoilage and pathogenic bacteria during processing and subsequent storage of ready-to-eat shrimp (*Pandalus borealis*). *International Journal of Food Microbiology* **157:** 16-27.

Merlin C, Mahillon J, Nešvera J, Toussaint A (2000). Gene recruiters and transporters: the modular structure of bacterial mobile elements, p. 363-409. In Thomas CM (Ed.), *The horizontal gene pool*. Hardwood Academic Publishers, Amsterdam, The Netherlands.

Michael GB, Cardoso M, Schwarz S (2005). Class 1 integron-associated gene cassettes in *Salmonella enterica* subsp *enterica* serovar Agona isolated from pig carcasses in Brazil. *Journal of Antimicrobial Chemotherapy* **55:** 776-779.

Michael GB, Butaye P, Cloeckaert A, Schwarz S (2006). Genes and mutations conferring antimicrobial resistance in *Salmonella*: an update. *Microbes and Infection* **8:** 1898-1914.

Modrie P, Beuls E, Mahillon J (2010). Differential transfer dynamics of pAW63 plasmid among members of the *Bacillus cereus* group in food microcosms. *Journal of Applied Microbiology* **108:** 888-897.

Mølbak K (2005). Human health consequences of antimicrobial drug-resistant *Salmonella* and other foodborne pathogens. *Clinical Infectious Diseases* **41:** 1613-1620.

Molin S, Tolker-Nielsen T (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Current Opinion in Biotechnology* **14:** 255-261.

Molina L, Udaondo Z, Duque E, Fernández M, Molina-Santiago C, Roca A, Porcel M, de la Torre J, Segura A, Plesiat P, Jeannot K, Ramos JL (2014). Antibiotic resistance determinants in a *Pseudomonas putida* strain isolated from a hospital. *Plos One* **9:** e81604.

Montville TJ, Matthews KR (2007). Growth, survival, and death of microbes in foods. In Doyle MP and Beuchat LR (Eds.) *Food Microbiology - Fundamentals and Frontiers.* Washington, USA, ASM Press.

Moore JE, Rao JR, Moore PJA, Millar BC, Goldsmith CE, Loughrey A, Rooney PJ (2010). Determination of total antibiotic resistance in waterborne bacteria in rivers and streams in Northern Ireland: Can antibiotic-resistant bacteria be an indicator of ecological change? *Aquatic Ecology* **44:** 349-358.

Morvan A, Moubareck C, Leclercq A, Hervé-Bazin M, Bremont S, Lecuit M, Courvalin P, Le Monnier A (2010). Antimicrobial resistance of *Listeria monocytogenes* strains isolated from humans in France. *Antimicrobial Agents and Chemotherapy* **54:** 2728-2731.

Musovic S, Oregaard G, Kroer N, Sørensen SJ (2006). Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among Gram-positive and Gram-negative bacteria indigenous to the barley rhizosphere. *Applied and Environmental Microbiology* **72:** 6687-6692.

Muyzer G, de Waal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRna. *Applied and Environmental Microbiology* **59:** 695-700.

Nagachinta S, Chen JR (2009). Integron-mediated antibiotic resistance in Shiga toxin-producing *Escherichia coli*. *Journal of Food Protection* **72:** 21-27.

Nataro JP, Kaper JB (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews* **11:** 142-201.

Nguyen KT, Piastro K, Gray TA, Derbyshire KM (2010). Mycobacterial biofilms facilitate horizontal DNA transfer between strains of *Mycobacterium smegmatis*. *Journal of Bacteriology* **192:** 5134-5142.

Nielsen KM, Johnsen PJ, Bensasson D, Daffonchio D (2007). Release and persistence of extracellular DNA in the environment. *Environmental Biosafety Research* **6:** 37-53.

Niewold TA (2007). The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poultry Science* **86:** 605-609.

Nógrády N, Gadó I, Tóth A, Pászti J (2005). Antibiotic resistance and class 1 integron patterns of non-typhoidal human *Salmonella* serotypes isolated in Hungary in 2002 and 2003. *International Journal of Antimicrobial Agents* **26:** 126-132.

Nordstrom L, Liu CM, Price LB (2013). Foodborne urinary tract infections: a new paradigm for antimicrobial-resistant foodborne illness. *Frontiers in Microbiology* **4:** 29.

Ohlsson T (1994). Minimal processing - preservation methods of the future: an overview. *Trends in Food Science & Technology* **5:** 341-344.

OJEC (1998). Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market. *Official Journal of the European Communities* 24/04/1998 **L123:** 1-63.

OJEC (2000). Commission Decision of 22 December 1999 on the communicable diseases to be progressively covered by the Community network under Decision No 2119/98/EC of the European Parliament and of the Council. *Official Journal of the European Communities* 03/02/2000 **L28:** 50-53.

OJEC (2002). Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal of the European Communities* **L31:** 1-24.

OJEU (2003a). Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union* **L268:** 29-43.

OJEU (2003b). Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. *Official Journal of the European Union* **L325:** 31-40.

OJEU (2004a). Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. *Official Journal of the European Union* **L139:** 1-54.

OJEU (2004b). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs. *Official Journal of the European Union* **L139:** 55-205.

OJEU (2004c). Corrigendum to Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Official Journal of the European Union* **L226:** 83-127.

OJEU (2005a). Commission Regulation (EC) No 1003/2005 of 30 June 2005 implementing Regulation (EC) No 2160/2003 as regards a Community target for the reduction of the prevalence of certain salmonella serotypes in breeding flocks of *Gallus gallus* and amending Regulation (EC) No 2160/2003. *Official Journal of the European Union* **L170:** 12-17.

OJEU (2005b). Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union* **L338:** 1-26.

Oliver JD, McDougald D, Barrett T, Glover LA, Prosser JI (1995). Effect of temperature and plasmid carriage on nonculturability in organisms targeted for release. *Fems Microbiology Ecology* **17:** 229-237.

Pagès JM, James CE, Winterhalter M (2008). The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nature Reviews Microbiology* **6:** 893-903.

Palacios I, Moro C, Lozano M, D'Arrigo M, Guillamón E, Garcia-Lafuente A, Villares A (2011). Use of modified atmosphere packaging to preserve mushroom quality during storage. *Recent Patents on Food, Nutrition & Agriculture* **3:** 196-203.

Panos GZ, Betsi GI, Falagas ME (2006). Systematic review: are antibiotics detrimental or beneficial for the treatment of patients with *Escherichia coli* O157:H7 infection? *Alimentary Pharmacology & Therapeutics* **24:** 731-742.

Partridge SR, Tsafnat G, Coiera E, Iredell JR (2009). Gene cassettes and cassette arrays in mobile resistance integrons. *Fems Microbiology Reviews* **33:** 757-784.

Perrin M, Bemer M, Delamare C (2003). Fatal case of *Listeria innocua* bacteremia. *Journal of Clinical Microbiology* **41:** 5308-5309.

Phillips CA (1996). Review: Modified atmosphere packaging and its effects on the microbiological quality and safety of produce. *International Journal of Food Science and Technology* **31:** 463-479.

Picozzi C, Volponi G, Vigentini I, Grassi S, Foschino R (2012). Assessment of transduction of *Escherichia coli* Stx2-encoding phage in dairy process conditions. *International Journal of Food Microbiology* **153:** 388-394.

Poole K (2004). Resistance to β-lactam antibiotics. *Cellular and Molecular Life Sciences* **61:** 2200-2223.

Poole K (2005). Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy* **56:** 20-51.

Pourshaban M, Ferrini AM, Mannoni V, Oliva B, Aureli P (2002). Transferable tetracycline resistance in *Listeria monocytogenes* from food in Italy. *Journal of Medical Microbiology* **51:** 564-566.

Povilonis J, Šeputienė V, Ružauskas M, Šiugždinienė R, Virgailis M, Pavilonis A, Sužiedėlienė E (2010). Transferable Class 1 and 2 Integrons in *Escherichia coli* and *Salmonella enterica* Isolates of Human and Animal Origin in Lithuania. *Foodborne Pathogens and Disease* **7:** 1185-1192.

Powers JH (2004). Antimicrobial drug development - the past, the present, and the future. *Clinical Microbiology and Infection* **10:** 23-31.

Ramirez MS, Tolmasky ME (2010). Aminoglycoside modifying enzymes. *Drug Resistance Updates* **13:** 151-171.

Ramos S, Silva N, Caniça M, Capelo-Martinez JL, Brito F, Igrejas G, Poeta P (2013). High prevalence of antimicrobial-resistant *Escherichia coli* from animals at slaughter: a food safety risk. *Journal of the Science of Food and Agriculture* **93:** 517-526.

Reisner A, Höller BM, Molin S, Zechner EL (2006). Synergistic effects in mixed *Escherichia coli* biofilms: Conjugative plasmid transfer drives biofilm expansion. *Journal of Bacteriology* **188:** 3582-3588.

Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ (2006). Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7 *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathogens and Disease* **3:** 59-67.

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003). Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends in Microbiology* **11:** 94-100.

Riemann L, Steward GF, Fandino LB, Campbell L, Landry MR, Azam F (1999). Bacterial community composition during two consecutive NE Monsoon periods in the Arabian Sea studied by denaturing gradient gel electrophoresis (DGGE) of rRNA genes. *Deep-Sea Research II* **46:** 1791-1811.

Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA, Cohen ML (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine* **308:** 681-685.

Rizzotti L, La Gioia F, Dellaglio F, Torriani S (2009). Molecular diversity and transferability of the tetracycline resistance gene *tet*(M), carried on Tn*916-1545* family transposons, in enterococci from a total food chain. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **96:** 43-52.

Roberts AP, Chandler M, Courvalin P, Guédon G, Mullany P, Pembroke T, Rood JI, Smith CJ, Summers AO, Tsuda M, Berg DE (2008). Revised nomenclature for transposable genetic elements. *Plasmid* **60:** 167-173.

Rocourt J, Hof H, Schrettenbrunner A, Malinverni R, Bille J (1986). Acute purulent *Listeria seelingeri* meningitis in an immunocompetent adult. Schweizerische *Medizinische Wochenschrift* **116:** 248-251.

Røder HL, Hansen LH, Sørensen SJ, Burmølle M (2013). The impact of the conjugative IncP-1 plasmid pKJK5 on multispecies biofilm formation is dependent on the plasmid host. *Fems Microbiology Letters* **344:** 186-192.

Rodríguez-Rojas A, Rodríguez-Beltran J, Couce A, Blázquez J (2013). Antibiotics and antibiotic resistance: a bitter fight against evolution. *International Journal of Medical Microbiology* **303:** 293-297.

Rosengren LB, Waldner CL, Reid-Smith RJ (2009). Associations between antimicrobial resistance phenotypes, antimicrobial resistance genes, and virulence genes of fecal *Escherichia coli* isolates from healthy grow-finish pigs. *Applied and Environmental Microbiology* **75:** 1373-1380.

Ross RP, Morgan S, Hill C (2002). Preservation and fermentation: past, present and future. *International Journal of Food Microbiology* **79:** 3-16.

Ruiz J (2003). Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy* **51:** 1109-1117.

Rumbo C, Fernández-Moreira E, Merino M, Poza M, Mendez JA, Soares NC, Mosquera A, Chaves F, Bou G (2011). Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy* **55:** 3084-3090.

Ryu SH, Park SG, Choi SM, Hwang YO, Ham HJ, Kim SU, Lee YK, Kim MS, Park GY, Kim KS, Chae YZ (2012). Antimicrobial resistance and resistance genes in *Escherichia coli* strains isolated from commercial fish and seafood. *International Journal of Food Microbiology* **152:** 14-18.

References

Sáenz Y, Briñas L, Domínguez E, Ruiz J, Zarazaga M, Vila J, Torres C (2004). Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. *Antimicrobial Agents and Chemotherapy* **48:** 3996-4001.

Sáenz Y, Vinué L, Ruiz E, Somalo S, Martínez S, Rojo-Bezares B, Zarazaga M, Torres C (2010). Class 1 integrons lacking *qacE*Δ1 and *sul1* genes in *Escherichia coli* isolates of food, animal and human origins. *Veterinary Microbiology* **144:** 493-497.

Salimnia H, Patel D, Lephart PR, Fairfax MR, Chandrasekar PH (2010). *Listeria grayi*: vancomycin-resistant, gram-positive rod causing bacteremia in a stem cell transplant recipient. *Transplant Infectious Disease* **12:** 526-528.

Samelis J, Kakouri A, Georgiadou KG, Metaxopoulos J (1998). Evaluation of the extent and type of bacterial contamination at different stages of processing of cooked ham. *Journal of Applied Microbiology* **84:** 649-660.

Sánchez-Vargas FM, Abu-El-Haija MA, Gómez-Duarte OG (2011). *Salmonella* infections: an update on epidemiology, management, and prevention. *Travel Medicine and Infectious Disease* **9:** 263-277.

Sandasi M, Leonard CM, Viljoen AM (2008). The effect of five common essential oil components on *Listeria monocytogenes* biofilms. *Food Control* **19:** 1070-1075.

Sarowska J, Drulis-Kawa Z, Guz K, Jankowski S, Wojnicz D (2009). Conjugative transfer of plasmid encoding extended-spectrum beta-lactamase to recipient *Salmonella* strains. *Advances in Clinical and Experimental Medicine* **18:** 63-70.

Savage VJ, Chopra I, O'Neill AJ (2013). *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. *Antimicrobial Agents and Chemotherapy* **57:** 1968-1970.

SCENIHR (2009). Assessment of the antibiotic resistance effects of biocides. Scientific Committee on Emerging and Newly Identified Health Risks, 19 January 2009. Available at: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf. Date last accessed: April 11, 2014.

Schauer M, Massana R, Pedrós-Alió C (2000). Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. *Fems Microbiology Ecology* **33:** 51-59.

Schjørring S, Krogfelt KA (2011). Assessment of bacterial antibiotic resistance transfer in the gut. *International Journal of Microbiology* **2011:** 312956.

Schlech WF, Lavigne PM, Bortolussi RA, Allen AC, Haldane EV, Wort AJ, Hightower AW, Johnson SE, King SH, Nicholls ES, Broome CV (1983). Epidemic listeriosis - evidence for transmission by food. *The New England Journal of Medicine* **308:** 203-206.

Schlüter A, Heuer H, Szczepanowski R, Forney LJ, Thomas CM, Pühler A, Top EM (2003). The 64 508 bp IncP-1β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1β group. *Microbiology* **149:** 3139-3153.

Schröder G, Lanka E (2005). The mating pair formation system of conjugative plasmids-A versatile secretion machinery for transfer of proteins and DNA. *Plasmid* **54:** 1-25.

Seitz P, Blokesch M (2013). Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. *Fems Microbiology Reviews* **37:** 336-363.

Settanni L, Corsetti A (2008). Application of bacteriocins in vegetable food biopreservation. *International Journal of Food Microbiology* **121:** 123-138.

Settanni L, Miceli A, Francesca N, Cruciata M, Moschetti G (2013). Microbiological investigation of *Raphanus sativus* L. grown hydroponically in nutrient solutions contaminated with spoilage and pathogenic bacteria. *International Journal of Food Microbiology* **160:** 344-352.

Shi X, Zhu X (2009). Biofilm formation and food safety in food industries. *Trends in Food Science & Technology* **20:** 407-413.

Shintani M, Matsui K, Inoue J, Hosoyama A, Ohji S, Yamazoe A, Nojiri H, Kimbara K, Ohkuma M (2014). Single-cell analyses revealed transfer ranges of IncP-1, IncP-7, and IncP-9 plasmids in a soil bacterial community. *Applied and Environmental Microbiology* **80:** 138-145.

Silk BJ, Mahon BE, Griffin PM, Gould H, Tauxe RV, Crim SM, Jackson KA, Gerner-Smidt P, Herman KM, Henao OL (2013). Vital signs: *Listeria* illnesses, deaths, and outbreaks - United States, 2009-2011. *Morbidity and Mortality Weekly Report* **62:** 448-452.

Silva N, Costa L, Gonçalves A, Sousa M, Radhouani H, Brito F, Igrejas G, Poeta P (2012). Genetic characterisation of extended-spectrum -lactamases in *Escherichia coli* isolated from retail chicken products including CTX-M-9 containing isolates: a food safety risk factor. *British Poultry Science* **53:** 747-755.

Simões M, Simões LC, Vieira MJ (2010). A review of current and emergent biofilm control strategies. *Lwt-Food Science and Technology* **43:** 573-583.

Singh R, Schroeder CM, Meng J, White DG, McDermott PF, Wagner DD, Yang H, Simjee S, DebRoy C, Walker RD, Zhao S (2005). Identification of antimicrobial resistance and class 1 integrons in Shiga toxin-producing *Escherichia coli* recovered from humans and food animals. *Journal of Antimicrobial Chemotherapy* **56:** 216-219.

Singh P, Wani AA, Karim AA, Langowski HC (2012). The use of carbon dioxide in the processing and packaging of milk and dairy products: a review. *International Journal of Dairy Technology* **65:** 161-177.

Singleton P, Anson AE (1981). Conjugal transfer of R-plasmid R1*drd-19* in *Escherichia coli* below 22°C. *Applied and Environmental Microbiology* **42:** 789-791.

Sintim HO, Al Smith J, Wang JX, Nakayama S, Yan L (2010). Paradigm shift in discovering next-generation anti-infective agents: targeting quorum sensing, c-di-GMP signaling and biofilm formation in bacteria with small molecules. *Future Medicinal Chemistry* **2:** 1005-1035.

Sivertsvik M, Jeksrud WK, Rosnes JT (2002). A review of modified atmosphere packaging of fish and fishery products - significance of microbial growth, activities and safety. *International Journal of Food Science and Technology* **37:** 107-127.

Skippington E, Ragan MA (2011). Lateral genetic transfer and the construction of genetic exchange communities. *Fems Microbiology Reviews* **35:** 707-735.

References

Skurnik D, Le Menac'h A, Zurakowski D, Mazel D, Courvalin P, Denamur E, Andremont A, Ruimy R (2005). Integron-associated antibiotic resistance and phylogenetic grouping of *Escherichia coli* isolates from healthy subjects free of recent antibiotic exposure. *Antimicrobial Agents and Chemotherapy* **49:** 3062-3065.

Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Herman L, Haesebrouck F, Butaye P (2010). Broad-spectrum β-lactamases among *Enterobacteriaceae* of animal origin: molecular aspects, mobility and impact on public health. *Fems Microbiology Reviews* **34:** 295-316.

Smet A, Rasschaert G, Martel A, Persoons D, Dewulf J, Butaye P, Catry B, Haesebrouck F, Herman L, Heyndrickx M (2011). *In situ* ESBL conjugation from avian to human *Escherichia coli* during cefotaxime administration. *Journal of Applied Microbiology* **110:** 541-549.

Smith JL, Fratamico PM, Gunther NW (2007). Extraintestinal pathogenic *Escherichia coli*. *Foodborne Pathogens and Disease* **4:** 134-163.

Smith KE, Wilker PR, Reiter PL, Hedican EB, Bender JB, Hedberg CW (2012). Antibiotic treatment of *Escherichia coli* O157 infection and the risk of hemolytic uremic syndrome, Minnesota. *Pediatric Infectious Disease Journal* **31:** 37-41.

Sofos JN, Geornaras I (2010). Overview of current meat hygiene and safety risks and summary of recent studies on biofilms, and control of *Escherichia coli* O157:H7 in nonintact, and *Listeria monocytogenes* in ready-to-eat, meat products. *Meat Science* **86:** 2-14.

Soni KA, Nannapaneni R (2010). Removal of *Listeria monocytogenes* biofilms with bacteriophage P100. *Journal of Food Protection* **73:** 1519-1524.

Sørensen SJ, Sørensen AH, Hansen LH, Oregaard G, Veal D (2003). Direct detection and quantification of horizontal gene transfer by using flow cytometry and *gfp* as a reporter gene. *Current Microbiology* **47:** 129-133.

Sørensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S (2005). Studying plasmid horizontal transfer *in situ*: a critical review. *Nature Reviews. Microbiology* **3:** 700-710.

Soufi L, Sáenz Y, Vinué L, Abbassi MS, Ruiz E, Zarazaga M, Ben Hassen A, Hammami S, Torres C (2011). Escherichia coli of poultry food origin as reservoir of sulphonamide resistance genes and integrons. *International Journal of Food Microbiology* **144:** 497-502.

Speranza B, Corbo MR, Sinigaglia M (2011). Effects of nutritional and environmental conditions on *Salmonella* sp. biofilm formation. *Journal of Food Science* **76:** M12-16.

Stanton TB, Humphrey SB, Sharma VK, Zuerner RL (2008). Collateral effects of antibiotics: Carbadox and metronidazole induce VSH-1 and facilitate gene transfer among *Brachyspira hyodysenterae* strains. *Applied and Environmental Microbiology* **74:** 2950-2956.

Stecher B, Denzler R, Maier L, Bernet F, Sanders MJ, Pickard DJ, Barthel M, Westendorf AM, Krogfelt KA, Walker AW, Ackermann M, Dobrindt U, Thomson NR, Hardt WD (2012). Gut inflammation can boost horizontal gene transfer between pathogenic and commensal *Enterobacteriaceae*. *Proceedings of the National Academy of Sciences of the United States of America* **109:** 1269-1274.

Stewart PS (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology* **292:** 107-113.

Stockwell VO, Duffy B (2012). Use of antibiotics in plant agriculture. *Revue scientifique et technique* **31:** 199-210.

Stokes HW, Hall RM (1989). A novel family of potentially mobile dna elements encoding site-specific gene-integration functions: integrons. *Molecular Microbiology* **3:** 1669-1683.

Stokes HW, Gillings MR (2011). Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. *Fems Microbiology Reviews* **35:** 790-819.

Straub JA, Hertel C, Hammes WP (1999). The fate of recombinant DNA in thermally treated fermented sausages. *European Food Research and Technology* **210:** 62-67.

Su LH, Chiu CH, Chu C, Ou JT (2004). Antimicrobial resistance in nontyphoid *Salmonella* serotypes: a global challenge. *Clinical Infectious Diseases* **39:** 546-551.

Sunde M (2005). Prevalence and characterization of class 1 and class 2 integrons in *Escherichia coli* isolated from meat and meat products of Norwegian origin. *Journal of Antimicrobial Chemotherapy* **56:** 1019-1024.

Sunde M, Norström M (2005). The genetic background for streptomycin resistance in *Escherichia coli* influences the distribution of MICs. *Journal of Antimicrobial Chemotherapy* **56:** 87-90.

Szmolka A, Nagy B (2013). Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. *Frontiers in Microbiology* **4:** 258.

Thomas CM, Nielsen KM (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews Microbiology* **3:** 711-721.

Threlfall EJ, Ward LR, Frost JA, Willshaw CA (2000). The emergence and spread of antibiotic resistance in food-borne bacteria. *International Journal of Food Microbiology* **62:** 1-5.

Toleman MA, Bennett PM, Walsh TR (2006). IS*CR* elements: novel gene-capturing systems of the 21st century? *Microbiology and Molecular Biology Reviews* **70:** 296-316.

Tollefson L, Karp BE (2004). Human health impact from antimicrobial use in food animals. *Medecine Et Maladies Infectieuses* **34:** 514-521.

Tompkin RB (2002). Control of *Listeria monocytogenes* in the food-processing environment. *Journal of Food Protection* **65:** 709-725.

Toomey N, Monaghan A, Fanning S, Bolton DJ (2009a). Assessment of antimicrobial resistance transfer between lactic acid bacteria and potential foodborne pathogens using *in vitro* methods and mating in a food matrix. *Foodborne Pathogens and Disease* **6:** 925-933.

Toomey N, Monaghan A, Fanning S, Bolton DJ (2009b). Assessment of horizontal gene transfer in Lactic acid bacteria - a comparison of mating techniques with a view to optimising conjugation conditions. *Journal of Microbiological Methods* **77:** 23-28.

Tupin A, Gualtieri M, Roquet-Banères F, Morichaud Z, Brodolin K, Leonetti JP (2010). Resistance to rifampicin: at the crossroads between ecological, genomic and medical concerns. *International Journal of Antimicrobial Agents* **35:** 519-523.

References

Uyttendaele M, De Troy P, Debevere J (1999). Incidence of *Listeria monocytogenes* in different types of meat products on the Belgian retail market. *International Journal of Food Microbiology* **53:** 75-80.

Uyttendaele M, Busschaert P, Valero A, Geeraerd AH, Vermeulen A, Jacxsens L, Goh KK, De Loy A, Van Impe JF, Devlieghere F (2009). Prevalence and challenge tests of Listeria monocytogenes in Belgian produced and retailed mayonnaise-based deli-salads, cooked meat products and smoked fish between 2005 and 2007. *International Journal of Food Microbiology* **133:** 94-104.

Uyttendaele M, Jacxsens L, De Loy-Hendrickx A, Devlieghere F, Debevere J (2010) *Microbiologische richtwaarden en wettelijke criteria*. Uyttendaele et al. 2010, LFMFP-UGent, Ghent, Belgium.

Van Coillie E, Werbrouck H, Heyndrickx M, Herman L, Rijpens N (2004). Prevalence and typing of *Listeria monocytogenes* in ready-to-eat food products on the Belgian market. *Journal of Food Protection* **67:** 2480-2487.

van den Eede G, Aarts HJ, Buhk HJ, Corthier G, Flint HJ, Hammes W, Jacobsen B, Midtvedt T, van der Vossen J, von Wright A, Wackernagel W, Wilcks A (2004). The relevance of gene transfer to the safety of food and feed derived from genetically modified (GM) plants. *Food and Chemical Toxicology* **42:** 1127-1156.

Van der Auwera GA, Timmery S, Hoton F, Mahillon J (2007). Plasmid exchanges among members of the *Bacillus cereus* group in foodstuffs. *International Journal of Food Microbiology* **113:** 164-172.

van der Veen S, Abee T (2010). Dependence of continuous-flow biofilm formation by *Listeria monocytogenes* EGD-e on SOS response factor YneA. *Applied and Environmental Microbiology* **76:** 1992-1995.

van der Veen S, Abee T (2011). Bacterial SOS response: a food safety perspective. *Current Opinion in Biotechnology* **22:** 136-142.

van Elsas JD, Bailey MJ (2002). The ecology of transfer of mobile genetic elements. *Fems Microbiology Ecology* **42:** 187-197.

van Essen-Zandbergen A, Smith H, Veldman K, Mevius D (2007). Occurrence and characteristics of class 1, 2 and 3 integrons in *Escherichia coli*, *Salmonella* and *Campylobacter* spp. in the Netherlands. *Journal of Antimicrobial Chemotherapy* **59:** 746-750.

Van Meervenne E, Van Coillie E, Kerckhof FM, Devlieghere F, Herman L, De Gelder LS, Top EM, Boon N (2012). Strain-specific transfer of antibiotic resistance from an environmental plasmid to foodborne pathogens. *Journal of Biomedicine and Biotechnology* **2012:** 834598.

Varga M, Kuntová L, Pantůček R, Mašlaňová I, Růžičková V, Doškař J (2012). Efficient transfer of antibiotic resistance plasmids by transduction within methicillin-resistant *Staphylococcus aureus* USA300 clone. *Fems Microbiology Letters* **332:** 146-152.

Vasilopoulos C, De Maere H, De Mey E, Paelinck H, De Vuyst L, Leroy F (2010). Technology-induced selection towards the spoilage microbiota of artisan-type cooked ham packed under modified atmosphere. *Food Microbiology* **27:** 77-84.

Venturini C, Beatson SA, Djordjevic SP, Walker MJ (2010). Multiple antibiotic resistance gene recruitment onto the enterohemorrhagic *Escherichia coli* virulence plasmid. *FASEB journal* **24:** 1160-1166.

Vermeiren L, Devlieghere F, Debevere J (2004). Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. *International Journal of Food Microbiology* **96:** 149-164.

Verraes C, Van Boxstael S, Van Meervenne E, Van Coillie E, Butaye P, Catry B, de Schaetzen MA, Van Huffel X, Imberechts H, Dierick K, Daube G, Saegerman C, De Block J, Dewulf J, Herman L (2013). Antimicrobial resistance in the food chain: a review. *International Journal of Environmental Research and Public Health* **10:** 2643-2669.

Vogel RF, Becke-schmid M, Entgens P, Gaier W, Hammes WP (1992). Plasmid transfer and segregation in *Lactobacillus curvatus* LTH1432 *in vitro* and during sausage fermentations. *Systematic and Applied Microbiology* **15:** 129-136.

Wainwright M (2004). Hitler's penicillin. *Perspectives in Biology and Medicine* **47:** 189-198.

Walsh C, Duffy G, Nally P, O'Mahony R, McDowell DA, Fanning S (2008). Transfer of ampicillin resistance from *Salmonella Typhimurium* DT104 to *Escherichia coli* K12 in food. *Letters in Applied Microbiology* **46:** 210-215.

Walsh C, Fanning S (2008). Antimicrobial resistance in foodborne pathogens - a cause for concern? *Current Drug Targets* **9:** 808-815.

Walsh TR, Weeks J, Livermore DM, Toleman MA (2011). Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infectious Diseases*.

Walsh CT, Wencewicz TA (2014). Prospects for new antibiotics: a molecule-centered perspective. *Journal of Antibiotics* **67:** 7-22.

Watanabe T (1963). Infective heredity of multiple drug resistance in bacteria. *Bacteriological Reviews* **27:** 87-115.

Wegener HC (2006). Risk management for the limitation of antibiotic resistance - experience of Denmark. *International Journal of Medical Microbiology* **296:** 11-13.

Weiss J, Ros-Chumillas M, Peña L, Egea-Cortines M (2007). Effect of storage and processing on plasmid, yeast and plant genomic DNA stability in juice from genetically modified oranges. *Journal of Biotechnology* **128:** 194-203.

Wells JG, Davis BR, Wachsmuth IK, Riley LW, Remis RS, Sokolow R, Morris GK (1983). Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *Journal of Clinical Microbiology* **18:** 512-520.

Werner G, Coque TM, Franz C, Grohmann E, Hegstad K, Jensen L, van Schaik W, Weaver K (2013). Antibiotic resistant enterococci-Tales of a drug resistance gene trafficker. *International Journal of Medical Microbiology* **303:** 360-379.

White PA, McIver CJ, Deng YM, Rawlinson WD (2000). Characterisation of two new gene cassettes, *aadA5* and *dfrA17*. *Fems Microbiology Letters* **182:** 265-269.

White PA, McIver CJ, Rawlinson WD (2001). Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy* **45:** 2658-2661.

White DG, Zhao S, McDermott PF, Ayers S, Gaines S, Friedman S, Wagner DD, Meng J, Needle D, Davis M, DebRoy C (2002). Characterization of antimicrobial resistance among *Escherichia coli* O111 isolates of animal and human origin. *Microbial Drug Resistance-Mechanisms Epidemiology and Disease* **8:** 139-146.

References

Whitfield FB, Jensen N, Shaw KJ (2000). Role of *Yersinia intermedia* and *Pseudomonas putida* in the development of a fruity off-flavour in pasteurized milk. *The Journal of Dairy Research* **67:** 561-569.

WHO (2014). Antimicrobial resistance: global report on surveillance 2014. Available at: http://www.who.int/drugresistance/documents/surveillancereport/en/. Date last accessed: June 5, 2014.

Winkelströter LK, Gomes BC, Thomaz MRS, Souza VM, De Martinis ECP (2011). *Lactobacillus sakei* 1 and its bacteriocin influence adhesion of *Listeria monocytogenes* on stainless steel surface. *Food Control* **22:** 1404-1407.

Woodford N, Ellington MJ (2007). The emergence of antibiotic resistance by mutation. *Clinical Microbiology and Infection* **13:** 5-18.

Zechner EL, de la Cruz F, Eisenbrandt R, Grahn AM, Koraimann G, Lanka E, Muth G, Pansegrau W, Thomas CM, Wilkins BM, Zatyka M (2000). Conjugative-DNA transfer processes, p. 87-174. In Thomas CM (Ed.), *The horizontal gene pool*. Hardwood Academic Publishers, Amsterdam, The Netherlands.

Zhang Y, LeJeune JT (2008). Transduction of bla_{CMY-2}, *tet*(A), and *tet*(B) from *Salmonella enterica* subspecies *enterica* serovar Heidelberg to *S.* Typhimurium. *Veterinary Microbiology* **129:** 418-425.

Zhao S, White DG, Ge B, Ayers S, Friedman S, English L, Wagner D, Gaines S, Meng J (2001). Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. *Applied and Environmental Microbiology* **67:** 1558-1564.

Zottola EA, Sasahara KC (1994). Microbial biofilms in the food processing industry - should they be a concern? *International Journal of Food Microbiology* **23:** 125-148.

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Publications in international peer reviewed journals

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Van Meervenne E, De Weirdt R, Van Coillie E, Devlieghere F, Herman L & Boon N. (2014). Biofilm models for the food industry: hot-spots for plasmid transfer? *Pathogens & Disease* **70:** 332-338.

Verraes C, Van Boxstael S, **Van Meervenne E**, Van Coillie E, Butaye P, Catry B, de Schaetzen MA, Van Huffel X, Imberechts H, Dierick K, Daube G, Saegerman C, De Block J, Dewulf J & Herman L. (2013). Antimicrobial Resistance in the Food Chain: A Review. *International Journal of Environmental Research and Public Health* **10:** 2643-2669.

Van Meervenne E, Boon N, Verstraete K, Devlieghere F, De Reu K, Herman L, Buvens G, Piérard D & Van Coillie E (2013). Integron characterization and typing of Shiga toxin-producing *Escherichia coli* isolates in Belgium. *Journal of Medical Microbiology* **62:**712-719.

Van Meervenne E, Van Coillie E, Kerckhof FM, Devlieghere F, Herman L, De Gelder LS, Top EM & Boon N (2012). Strain-specific transfer of antibiotic resistance from an environmental plasmid to foodborne pathogens. *Journal of Biomedicine and Biotechnology* **2012:** 834598. Epub 2012 Jun 27.

Bertrand S, **Van Meervenne E**, De Baere T, Vanhoof R, Collard JM, Ruckly C, Taha M & Carion F (2011). Detection of a geographical and endemic cluster of hyper-invasive meningococcal strains. *Microbes and Infections* **13:** 684-690.

Bertrand S, Dierick K, Heylen K, De Baere T, Pochet B, Robesyn E, Lokietek S, **Van Meervenne E**, Imberechts H, De Zutter L & Collard JM (2010). Lessons learned from the management of a national outbreak of *Salmonella* Ohio linked to pork meat processing and distribution. *Journal of food protection* **73:** 529-534.

Van Meervenne E, Botteldoorn N, Lokietek S, Vatlet M, Cupa A, Naranjo M, Dierick K & Bertrand S (2009). Turtle-associated *Salmonella* septicaemia and meningitis in a 2-month-old baby. *Journal of Medical Microbiology* **58:** 1379-1381.

Vrints M, Mairiaux E, **Van Meervenne E**, Collard JM & Bertrand S (2009). Surveillance of antibiotic susceptibility patterns among *Shigella sonnei* strains isolated in Belgium during the 18-year period 1990 to 2007. *Journal of clinical microbiology* **47:** 1379-1385.

Participations in (inter)national scientific conferences

Van Meervenne E, De Weirdt R, Van Coillie E, Devlieghere F, Herman L & Boon N. Plasmid transfer in biofilms from a food industry perspective. 19th National Symposium on Applied Biological Sciences, Gembloux, Belgium, 7 February, 2014. ***Oral*** *presentation.*

Van Meervenne E. Transfer van antibioticumresistentie tijdens voedselproductie en – bewaring. 18th Conference on Food Microbiology – BSFM, Brussels, Belgium, 12-13 September 2013. *Invited Speaker.*

Van Meervenne E, Van Coillie E, Devlieghere F, Herman L & Boon N. Plasmid transfer in biofilms from a food industry perspective. 3rd European Congress on Microbial Biofilms – Basic and Clinical Aspects – Eurobiofilms 2013, Ghent, Belgium, 9-12 September 2013. ***Poster*** *presentation.*

Van Meervenne E, Van Coillie E, Devlieghere F, Herman L & Boon N. Plasmid transfer in biofilms: A food industry perspective. 5th Congress of European Microbiologists – FEMS 2013, Leipzig, Germany, 21-25 July 2013. ***Poster*** *presentation.*

Van Meervenne E, Boon N, Verstraete K, Devlieghere F, De Reu K, Herman L, Buvens G, Piérard D & Van Coillie E. Integron characterization and typing of Belgian isolates of Shiga Toxin-Producing Escherichia coli. 5th Symposium on Antimicrobial Resistance in Animals and the Environment – ARAE2013, Ghent, Belgium, 30 June – 3 July 2013. ***Oral*** *presentation.*

Van Meervenne E, Van Coillie E, Devlieghere F & Boon N. Plasmid transfer in biofilms: Drip flow systems versus Flow through systems. 17th Conference on Food Microbiology – BSFM, Brussels, Belgium, 20-21 September 2012. ***Poster*** *presentation.*

Van Meervenne E, Van Coillie E, Kerckhof FM, Devlieghere F, Herman L, De Gelder L, Top E & Boon N. An environmental multiresistance plasmid can be transferred to foodborne pathogens. 23rd International ICFMH Symposium – FoodMicro 2012, Istanbul, Turkey, 3-6 September 2012. ***Oral*** *presentation.*

Van Meervenne E, Boon N, Verstraete K, Devlieghere F, De Reu K, Herman L, Buvens G, Piérard D & Van Coillie E. Integron characterization and typing of Belgian isolates of Shiga Toxin-Producing *Escherichia coli*. 23rd International ICFMH Symposium – FoodMicro 2012, Istanbul, Turkey, 3-6 September 2012. ***Poster*** *presentation.*

Van Meervenne E, Van Coillie E, Kerckhof FM, Devlieghere F, Herman L, De Gelder L, Top E & Boon N. An environmental multiresistance plasmid can be transferred to foodborne pathogens. 14th International Symposium on Microbial Ecology – ISME 2012, Copenhagen, Denmark, 19-24 August 2012. ***Poster*** *presentation.*

Van Meervenne E, Boon N, Verstraete K, Devlieghere F, De Reu K, Herman L, Buvens G, Piérard D & Van Coillie E. Integron characterization and typing of Belgian isolates of Shiga Toxin-Producing *Escherichia coli*. 14th International Symposium on Microbial Ecology – ISME 2012, Copenhagen, Denmark, 19-24 August 2012. ***Poster*** *presentation.*

Van Meervenne E, Van Coillie E, Devlieghere F, Herman L, De Gelder L, Top E & Boon N. An environmental multiresistance plasmid can be transferred to foodborne pathogens. 16th Conference on Food Microbiology – BSFM, Brussels, Belgium, 22-23 September 2011. ***Poster*** *presentation*

Van Meervenne E, Boon N, Verstraete K, Devlieghere F, Herman L, Piérard D & Van Coillie E. Integron Characterization of Shiga Toxin-Producing *Escherichia coli* in Belgium. 16th Conference on Food Microbiology – BSFM, Brussels, Belgium, 22-23 September 2011. ***Poster*** *presentation.*

Van Meervenne E, Van Coillie E, Devlieghere F & Boon N. Multiresistance plasmids can be transferred from an environmental strain to foodborne pathogens. 16th Symposium on Applied Biological Sciences, Ghent, Belgium, 20 December, 2010. ***Poster*** *presentation*

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